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(54) Title: METHOD FOR PREVENTING HIV-1 INFECTION OF CD4+ CELLS

(57) Abstract

This invention provides methods for inhibiting fusion of HIV–1 to CD4+ cells which comprise contacting CD4+ cells with a non–chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV–1 to the CD4+ cells is inhibited. This invention also provides methods for inhibiting HIV–1 infection of CD4+ cells which comprise contacting CD4+ cells with a non–chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV–1 to the CD4+ cells is inhibited, thereby inhibiting the HIV–1 infection. This invention provides non–chemokine agents capable of binding to the chemokine receptor and inhibiting fusion of HIV–1 to CD4+ cells. This invention also provides pharmaceutical compositions comprising an amount of the non–chemokine agent capable of binding to the chemokine receptor and inhibiting fusion of HIV–1 to CD4+ cells effective to prevent fusion of HIV–1 to CD4+ cells and a pharmaceutically acceptable carrier.

METHOD FOR PREVENTING HIV-1 INFECTION OF CD4+ CELLS

This application is a continuation-in-part application of U.S. Serial No. 08/876,078, filed June 13, 1997, the contents of which is hereby incorporated by reference.

5   Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic
10   citation for these references may be found at the end of each series of experiments.

Background of the Invention

15   Chemokines are a family of related soluble proteins of molecular weight between 8 and 10KDa, secreted by lymphocytes and other cells, which bind receptors on target cell surfaces resulting in the activation and mobilization of leukocytes, for example in the inflammatory process. Recently, Cocchi et al. demonstrated that the chemokines
20   RANTES, MIP-1α and MIP-1β are factors produced by CD8+ T lymphocytes which inhibit infection by macrophage-tropic primary isolates of HIV-1, but not infection by laboratory-adapted strains of the virus (1). These chemokines are members of the C-C group of chemokines, so
25   named because they have adjacent cysteine residues, unlike the C-X-C group which has a single amino acid separating these residues (2). While Cocchi et al. found that expression of HIV-1 RNA was suppressed by treatment with the chemokines, they did not identify the site of action of
30   these molecules.

A resonance energy transfer (RET) assay of HIV-1 envelope glycoprotein-mediated membrane fusion was used to determine whether fusion mediated by the envelope glycoprotein from
35   the primary macrophage-tropic isolate of HIV-1$_{JR-FL}$ would be specifically inhibited by chemokines, when compared with fusion mediated by the envelope glycoprotein from the

laboratory-adapted T lymphotropic strain $HIV-1_{LAI}$. As described below, it was demonstrated that this is indeed the case. This demonstrates that some chemokine receptors are fusion accessory molecules required for HIV-1 infection.
5 Previous studies have indicated that unidentified cell surface molecules are required for virus entry in addition to the HIV-1 receptor, CD4. While CD4 is required for HIV-1 attachment, the accessory molecules are required for the membrane fusion step of entry. These accessory molecules
10 are generally expressed only on human cells, so HIV-1 does not infect non-human CD4˙ cells (3-6). Moreover it is possible to complement non-human CD4˙ cells by fusing them (using polyethylene glycol) with CD4˙ human cells, resulting in a heterokaryon which is a competent target for HIV-1
15 envelope-mediated membrane fusion (7,8). These studies have been performed using laboratory-adapted T lymphotropic strains of the virus.

In some cases, it appears that fusion accessory molecules
20 are found on a subset of human CD4˙ cells and are required for infection by HIV-1 isolates with particular tropisms. For example, macrophage-tropic primary strains of HIV-1 such as $HIV-1_{JR-FL}$ may have different requirements for accessory molecules compared with laboratory-adapted T lymphotropic
25 strains such as $HIV-1_{LAI}$. This phenomenon may explain differences in tropism between HIV-1 strains.

The current invention comprises a series of new therapeutics for HIV-1 infection. It was demonstrated for the first time
30 that chemokines act at the fusion step of HIV-1 entry and specifically inhibit membrane fusion mediated by the envelope glycoprotein of primary macrophage-tropic primary viral isolates, not laboratory-adapted T lymphotrophic strains of the virus. Primary macrophage-tropic isolates of
35 the virus are of particular importance since they are the strains usually involved in virus transmission, and may have particular importance in the pathogenesis of HIV-1 infection.

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These results were obtained using a resonance energy transfer (RET) assay of HIV-1 envelope-mediated membrane fusion. Moreover, this assay is used to identify non-chemokines, including fragments of chemokines and modified chemokines, that inhibit HIV-1 envelope glycoprotein-mediated membrane fusion and thereby neutralize the virus, yet do not induce an inflammatory response.

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Summary of the Invention

This invention provides a method for inhibiting fusion of HIV-1 to CD4$^+$ cells which comprises contacting CD4 cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4$^+$ cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of CD4$^+$ cells which comprises contacting CD4$^-$ cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4$^+$ cells is inhibited, thereby inhibiting the HIV-1 infection.

This invention further provides non-chemokine agents capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4$^+$ cells.

This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide. In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonypeptidyl agent.

In addition, this invention provides pharmaceutical compositions comprising an amount of the above non-chemokine agents or agents capable of binding to fusin effective to inhibit fusion of HIV-1 to CD4$^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4$^+$ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the CD4$^+$ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the

other receptor.

This invention also provides a pharmaceutical composition comprising an amount of the above-described composition of matter effective to inhibit fusion of HIV-1 to CD4⁺ cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4⁺ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent.

This invention also provides a pharmaceutical composition comprising an amount of a composition of matter comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to CD4⁺ cells and a pharmaceutically acceptable carrier.

This invention provide methods for reducing the likelihood of HIV-1 infection in a subject comprising administering an above-described pharmaceutical composition to the subject. This invention also provides methods for treating HIV-1 infection in a subject comprising administering an above-described pharmaceutical composition to the subject.

This invention also provides methods for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a CD4⁺ cell which comprise: (a) contacting (i) a CD4⁺ cell which is labeled with a first dye and (ii) a cell expressing the HIV-1 envelope glycoprotein on its surface which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the CD4⁺ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the

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product of step (a) to conditions which would result in
resonance energy transfer if fusion has occurred; and (c)
determining whether there is a reduction of resonance energy
transfer, when compared with the resonance energy transfer
in the absence of the agent, a decrease in transfer
indicating that the agent is capable of inhibiting fusion of
HIV-1 to CD4⁺ cells.

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Brief Description of the Figures

Figure 1. Membrane fusion mediated by the HIV-1$_{JR-FL}$ envelope glycoprotein is inhibited by RANTES, MIP-1α and MIP-1β.

%RET resulting from the fusion of PM1 cells and HeLa-env$_{JR-FL}$ (■) or HeLa-env$_{LAI}$ (◆) was measured in the presence and absence of recombinant human chemokines at a range of concentrations: RANTES (80 - 2.5 ng/ml), MIP-1α (400 - 12.5 ng/ml) and MIP-1β (200 - 6.25 ng/ml), as indicated. Chemokines were added simultaneously with the cells at the initiation of a four hour incubation. Data are representative of more than three independent experiments which were run in duplicate. The percent inhibition of RET is defined as follows:

% Inhibition = 100 . [(Max RET - Min RET) - (Exp RET- Min RET)]/(Max RET - Min RET)

where Max RET is the %RET value obtained at four hours with HeLa-env cells and CD4-expressing cells in the absence of an inhibitory compound; Exp RET is the %RET value obtained for the same cell combination in the presence of an inhibitory compound and Min RET is the background %RET value obtained using HeLa cells in place of HeLa envelope-expressing cells.

Figure 2. CD4:HIV-1 gp120 binding in the presence of human chemokines.

The binding of soluble human CD4 to HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 was determined in an ELISA assay in the presence and absence of the monoclonal antibody OKT4A or recombinant human chemokines at

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a range of concentrations, identical to those used
in the RET inhibition studies of Figure 1: OKT4A
(62 - 0.3 nM), RANTES (10.3 - 0.3 nM), MIP-1α
(53.3 - 2.9 nM), and MIP-1β (25.6 - 0.8 nM).
Inhibitors were added simultaneously with
biotinylated HIV-1 gp120 to soluble CD4 coated
microtiter plates (Dynatech Laboratories, Inc.,
Chantilly, VA). Following a two hour incubation
at room temperature and extensive washing, an
incubation with streptavidin-horseradish
peroxidase was performed for one hour at room
temperature. Following additional washes,
substrate was added and the OD at 492 nm
determined in an ELISA plate reader. Data are
representative of two independent experiments
which were run in quadruplicate.

Figure 3. Specificity. time course and stage of ß-chemokine
inhibition of HIV-1 replication.

(a) PM1 cells (1 x10^6) were preincubated with
RANTES + MIP- 1α + MIP-1ß (R/Mα/Mß; 100ng/ml of
each) for 24h (-24h) or 2h (-2h), then washed
twice with phosphate buffered saline (PBS). HIV-1
(BaL env-complemented) virus (50ng of p24; see
legend to Table 1) was added for 2h, then the
cells were washed and incubated for 48h before
measurement of luciferase activity in cell lysates
as described previously (10,11). Alternatively,
virus and R/Mα/Mß were added simultaneously to
cells, and at the indicated time points (1h, 3h,
etc) the cells were washed twice in PBS,
resuspended in culture medium and incubated for
48h prior to luciferase assay. Time 0 represents
the positive control, to which no ß-chemokines
were added. +2h represents the mixture of virus
with cells for 2h prior to washing twice in PBS,
addition of R/Mα/Mß and continuation of the

culture for a further 48h before luciferase assay.

(b) PM1 cells (1x10^6) were infected with HIV-1 (500pg p24) grown in CEM cells (NL4/3; lanes 1-4) or macrophages (ADA; lanes 5-8), in the presence of 500ng/ml of RANTES (lanes 1 and 5) or MIP-1ß (lanes 2 and 6), or with no ß-chemokine (lanes 4 and 8). Lanes 3 and 7 are negative controls (no virus). All viral stocks used for the PCR assay were treated with DNAse for 30 min at 37°C, and tested for DNA contamination before use. After 2h, the cells were washed and resuspended in medium containing the same ß-chemokines for a further 8h. DNA was then extracted from infected cells using a DNA/RNA isolation kit (US Biochemicals). First round nested PCR was performed with primers: U3+, 5'-CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGG-3' (SEQ ID NO:1) preGag, 5'-AGCAAGCCGAGTCCTGCGTCGAGAG-3' (SEQ ID NO:2) and the second round with primers: LTR-test, 5'-GGGACTTTCCGCTGGGGACTTTC 3'(SEQ ID NO :3) LRC2, 5'-CCTGTTCGGGCGCCACTGCTAGAGATTTTCCAC 3' (SEQ ID NO:4) in a Perkin Elmer 2400 cycler with the following amplification cycles: 94°C for 5 min, 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s, 72°C for 7 min. M indicates 1kb DNA ladder; 1, 10, 100, 1000 indicate number of reference plasmid (pAD8) copies. The assay can detect 100 copies of reverse transcripts.

Figure 4: HIV-1 env-mediated membrane fusion of cells transiently expressing C-C CKR-5.

Membrane fusion mediated by ß-chemokine receptors expressed in HeLa cells was demonstrated as follows: Cells were transfected with control plasmid pcDNA3.1 or plasmid pcDNA3.1-CKR constructs using lipofectin (Gibco BRL). The

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pcDNA3.1 plasmid carries a T7-polymerase promoter and transient expression of ß-chemokine receptors was boosted by infecting cells with 1×10^7 pfu of vaccinia encoding the T7-polymerase (vFT7.3) 4h post-lipofection (9). Cells were then cultured overnight in R18-containing media and were tested for their ability to fuse with HeLa-JR-FL cells (filled columns) or HeLa-BRU cells (hatched column) in the RET assay. The %RET with control HeLa cells was between 3% and 4% irrespective of the transfected plasmid.

Figure 5   Membrane fusion mediated by the HIV_{LAI} envelope glycoprotein is inhibited by SDF-1.

% RET resulting from the fusion of PM1 cells and HeLa-env$_{JR-FL}$ or HeLa-env$_{LAI}$ cells (as indicated on the graph) was measured in the presence of recombinant SDF-1α (Gryphon Science, San Francisco) at the indicated concentrations. Experimental method as described in the legend to Fig. 1.

Figure 6.   Flow cytometric analysis of the binding of sCD4-gp120 complexes to (a) CCR5⁻ and (b) CCR5 L1.2 cells, a murine pre-B lymphoma line.

Cells are incubated for 15 min. with equimolar (~100nM) mixtures of sCD4 and biotinylated HIV-1$_{JR-FL}$ gp120 and then stained with a streptavidin-phycoerythrin conjugate, fixed with 2% paraformaldehyde, and analyzed by FACS. Cell number is plotted on the y-axis.

Figure 7.   Inhibition of HIV-1 envelope-mediated cell fusion by the bicyclam JM3100, measured using the RET assay, with the cell combinations indicated.

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Figure 8. Binding of CD4 and gp120 to CCR5.

Recombinant soluble CD4 (sCD4) and recombinant
gp120 were added in a range of concentrations
either individually or as an equimolar molecular
complex to recombinant L1.2 cells that express
human CCR5 on their cell surface. The recombinant
proteins were biotinylated as indicated. Binding
was detected by adding a streptavidin-
phycoerythrin conjugate and measuring the
fluoroescence emission at 590 nm following
excitation at 530 nm. The following species were
tested:

b-LAI:sCD4:   complex formed between scD4 and
               biotinylated HIV-1$_{LAI}$ gp120

b-JR-FL:sCD4: complex formed between sCD4 and
               biotinylated HIV-1$_{JR-FL}$ gp120

b-sCD4 alone: biotinylated sCD4 added in the
               absence of gp120

b-JR-FL alone: biotinylated HIV-1$_{JR-FL}$ gp120 added
               inthe absence of sCD4

These data demonstrate that complexation of
soluble CD4 and gp120 is necessary for CCR5
binding, as minimal binding is observed for sCD4
or gp120 alone. The data further demonstrate that
binding is observed for sCD4-gp120 complexes when
the gp120 is derived from macrophage-tropic (e.g.,
JR-FL) but not T cell-tropic (e.g., LAI) strains
of HIV, as expected from the known relationship
between HIV-1 tropism and co-receptor usage. All
data have been corrected for residual background
binding to nontransfected CCR5- L1.2 cells. To
enhance chemokine receptor expression, both
transfected and parental L1.2 cells were treated
with sodium butyrate prior to assay (Wu et al., J.
Exp. Med. 185:1681)

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Figure 9. The CCR5 Binding assay identifies and determines
the potency of inhibitors of the gp120-CCR5
interaction.

5

HIV-1 Inhibitory monoclonal antibodies were added
in a range of concentrations to reecmbinant L1.2
cells that express human CCR5 on their cell
surface and used to compete the binding of a

10   complex formed between sCD4 and biotinylated HIV-
1$_{JR-FL}$ gp 120, whose binding was detected using a
streptavidin-phycoerythrin conjugate. PA-8, -9, -
10, -11 and -12 are Progenics' monoclonal
antibodies that inhibit HIV-1 entry, while 2D7 is

15   a commercially available (Pharmingen, San Diego,
CA) ant-CCR5 monoclonal antibody that inhibits
HIV-1 entry. To enhance chemokine receptor
expression, both transfected and parental L1.2
cells were treated with sodium butyrate prior to

20   assay (Wu et al., J. Exp. Med. 185:1681).

Detailed Description of the Invention

This invention provides a method for inhibiting fusion of HIV-1 to CD4⁺ cells which comprises contacting CD4 cells
5   with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4⁺ cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of CD4⁺ cells which comprises contacting CD4⁻ cells
10   with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4⁺ cells is inhibited, thereby inhibiting the HIV-1 infection.

15   In this invention, a chemokine means RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection. A chemokine receptor means a receptor capable of binding RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection. Such chemokine receptor includes but not
20   limited to CCR5, CXCR4, CCR3 and CCR-2b.

Throughout this application, the receptor "fusin" is also named CXCR4 and the chemokine receptor C-C CKR5 is also named CCR5.
25

The HIV-1 used in this application unless specified will mean clinical or primary or field isolates or HIV-1 viruses which maintain their clinical characteristics. The HIV-1 clinical isolates may be passaged in primary peripheral
30   blood mononuclear cells. The HIV-1 clinical isolates may be macrophage-trophic.

The non-chemokine agents of this invention are capable of binding to chemokine receptors and inhibiting fusion of HIV-
35   1 to CD4⁺ cells. The non-chemokine agents include, but are not limited to, chemokine fragments and chemokine derivatives and analogues, but do not include naturally occurring chemokines. The non-chemokine agents include

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multimeric forms of the chemokine fragments and chemokine derivatives and analogues or fusion molecules which contain chemokine fragments, derivatives and analogues linked to other molecules.

5

The non-chemokine agents do not include bicyclams and their derivatives as described in U.S.Patent No. 5,021,409, issued June 4, 1991, the content of which is incorporated by reference into this application. Some bicyclam derivatives

10   have been previously described with antiviral activities (15, 16).

In an embodiment of this invention, the non-chemokine agent is an oligopeptide. In another embodiment, the non-

15   chemokine agent is a polypeptide. In still another embodiment, the non-chemokine agent is an antibody or a portion thereof. Antibodies against the chemokine receptor may easily be generated by routine experiments. It is also within the level of ordinary skill to synthesize fragments

20   of the antibody capable of binding to the chemokine receptor. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

Non-chemokine agents which are purely peptidyl in

25   composition can be either chemically synthesized by solid-phase methods (Merrifield, 1966) or produced using recombinant technology in either prokaryotic or eukaryotic systems. The synthetic and recombinant methods are well known in the art.

30

Non-chemokine agents which contain biotin or other nonpeptidyl groups can be prepared by chemical modification of synthetic or recombinant chemokines or non-chemokine agents. One chemical modification method involves periodate

35   oxidation of the 2-amino alcohol present on chemokines or non-chemokine agents possessing serine or threonine as their N-terminal amino acid (Geophegan and Stroh, 1992). The resulting aldehyde group can be used to link peptidyl or

non-peptidyl groups to the oxidized chemokine or non-chemokine agent by reductive amination, hydrazine, or other chemistries well known to those skilled in the art.

5   As used herein, a N-terminus of a protein should mean the terminus of the protein after it has been processed. In case of a secretory protein which contains a cleavable signal sequence, the N-terminus of a secretory protein should be the terminus after the cleavage of a signal
10   peptide.

This invention provides a method of identifying these non-chemokine agents. One way of identifying such agents, including non-peptidyl agents, that bind to a chemokine
15   receptor and inhibit fusion of HIV-1 to CD4$^+$ cells is to use the following assay: 1) Incubate soluble CD4 with biotinylated gp120 from HIV-1$_{JR-FL}$ or HIV-1$_{LAI}$; 2) Incubate this complex with CCR5 or CXCR4-expressing cells (for HIV-1$_{JR-FL}$ or HIV-1$_{LAI}$ gp120s, respectively) that do not express
20   CD4, in the presence of absence of a candidate inhibitor; 3) Wash and then incubate with streptavidin-phycoerythrin; and 4) Wash and then measure the amount of bound gp120 using a flow cytometer or fluorometer and calculate the degree of inhibition of binding by the inhibitor.
25

Alternative methods to detect bound gp120 can also be used in place of the biotinylated gp120-streptavidin-phycoerythrin method described above. For example, peroxidase-conjugated gp120 could be used in place of the
30   biotinylated gp120 and binding detected using an appropriate colorimetric substrate for peroxidase, with a spectrometric readout.

This invention further provides the non-chemokine agents
35   identified by the above methods.

This invention provides a non-chemokine agent capable of binding to the chemokine receptor and inhibiting fusion of

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HIV-1 to CD4⁺ cells with the proviso that the agent is not a known bicyclam or its known derivatives. In an embodiment, the non-chemokine is a polypeptide. In a further embodiment, this polypeptide is a fragment of the chemokine RANTES (Gong et al., 1996). In a still further embodiment, the polypeptide may also comprise the RANTES sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first eight N-terminal amino acids of the RANTES sequence (SEQ ID NO:5).

In a separate embodiment, the polypeptide may comprise the MIP-1ß sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first seven, eight, nine or ten N-terminal amino acids of the MIP-1ß sequence.

In another embodiment of non-chemokine agent, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by addition of an amino acid or oligopeptide. In a separate embodiment, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by removing the N-terminal alanine and replaced it by serine or threonine and additional amino acid or oligopeptide or nonpeptidyl moiety. In a further embodiment, the additional amino acid is methionine.

As described _infra_ in the section of Experimental Details, a cofactor for HIV-1 fusion and entry was identified and designated "fusin" (Feng et al., 1996). This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide.

In a further embodiment, the polypeptide comprises SDF-1 with deletion of the N-terminal amino acids of said sequence. The deletion may be the first six, seven, eight, or nine N-terminal amino acids of the SDF-1 sequence.

This invention also provides the above non-chemokine agent, wherein the polypeptide comprises SDF-1 sequence with the N-terminal sequence modified to produce antagonistic effect to SDF-1. One modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with biotin. Another modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with methionine. A further modification is to add the N-terminus of SDF-1 with a methionine before the terminal glycine.

In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonpeptidyl agent.

The agents capable of binding to fusin may be identified by screening different compounds for their capability to bind to fusin in vitro.

A suitable method has been described by Fowlkes, et al. (1994), international application number: PCT/US94/03143, international publication number: WO 94/23025, the content of which is incorporated by reference into this application. Briefly, yeast cells having a pheromone system are engineered to express a heterologous surrogate of a yeast pheromone system protein. The surrogate incorporates fusin and under some conditions performs in the pheromone system of the yeast cell a function naturally performed by the corresponding yeast pheromone system protein. Such yeast cells are also engineered to express a library of peptides whereby a yeast cell containing a peptide which binds fusin exhibits modulation of the interaction of surrogate yeast pheromone system protein with the yeast pheromone system and this modulation is a selectable or screenable event. Similar approaches may be used to identify agents capable of binding to both fusin and the chemokine receptor C-C CKR-5.

This invention also provides pharmaceutical compositions comprising an amount of such non-chemokine agents or agents

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capable of binding to fusin effective to inhibit fusion of HIV-1 to CD4⁺ cells and a pharmaceutically acceptable carrier.

5   Pharmaceutically acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol,
10   vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose,
15   dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example,
20   antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of
25   HIV-1 to CD4⁺ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the CD4⁺ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the
30   other receptor. In an embodiment, the cell surface receptor is CD4. In another embodiment, the ligand is an antibody or a portion of an antibody.

This invention also provides a pharmaceutical composition
35   comprising an amount of an above-described composition of matter effective to inhibit fusion of HIV-1 to CD4⁺ cells and a pharmaceutically acceptable carrier.

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This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4⁺ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol.

This invention also provides a pharmaceutical composition comprising an amount of a composition of matter comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to CD4⁺ cells and a pharmaceutically acceptable carrier.

This invention provide methods for reducing likelihood of HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject. This invention also provides methods for treating HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject.

This invention also provides methods for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a CD4⁺ cell which comprise: (a) contacting (i) a CD4⁺ cell which is labeled with a first dye and (ii) a cell expressing the HIV-1 envelope glycoprotein on its surface which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the CD4⁺ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is a reduction of resonance energy transfer, when compared with the resonance energy transfer in the absence of the agent, a decrease in transfer indicating that the agent is capable of inhibiting fusion of

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HIV-1 to CD4$^+$ cells.

HIV-1 only fuses with appropriate CD4$^+$ cells. For example, laboratory-adapted T lymphotropic HIV-1 strains fuse with most CD4$^+$ human cells. Clinical HIV-1 isolates do not fuse with most transformed CD4$^+$ human cell lines but do fuse with human primary CD4$^+$ cells such as CD4 T lymphocytes and macrophages. Routine experiments may be easily performed to determine whether the CD4$^+$ cell is appropriate for the above fusion assay.

As described in this invention, HIV-1 membrane fusion is monitored by a resonance energy transfer assay. The assay was described in the International Application Number, PCT/US94/14561, filed December 16, 1994 with International Publication Number WO 95/16789. This assay is further elaborated in a United States co-pending application no. 08/475,515, filed June 7, 1995. The contents of these applications are hereby incorporated by reference into this application.

In an embodiment of the above method, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the agent is an antibody or a portion thereof. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

In a separate embodiment, the CD4$^+$ cell is a PM1 cell. In another embodiment, the cell expressing the HIV-1 envelope glycoprotein is a HeLa cell expressing HIV-1$_{JR-FL}$ gp120/gp41.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) contacting an appropriate concentration of an agent with a chemokine receptor or a portion thereof under conditions permitting the binding of the agent to the chemokine receptor; (b) contacting the chemokine receptor

resulting from step (a) with a gp120/CD4 complex under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (c)
5   measuring the amount of bound gp120/CD4 complex wherein a decrease in the amount compared with the amount determined in the absence of the agent indicates that the agent is capable of inhibiting HIV-1 infection.

As used herein, the portion of the chemokine receptor used
10   in the above method is the portion which maintains the capability of binding to HIV, i.e. capable of interaction with the gp120/CD4 complex. It is the intention of this invention to cover hybrid molecules or genetically engineered molecules which comprise this portion or domain
15   of the chemokine receptor.

The gp120/CD4 complex used in the assay may include a truncated form of either molecules or hybrid proteins of molecules as long as the domain for binding to the chemokine
20   receptor is retained.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a chemokine receptor on a solid matrix;
25   (b) contacting the agent with the fixed chemokine receptor under conditions permitting the binding of the agent to the chemokine receptor; (c) removing the unbound agent; (d) contacting the fixed chemokine receptor resulting in step (c) with a gp120 in the presence of CD4 under conditions
30   permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (e) measuring the amount of bound gp120/CD4 complex; and (f) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the
35   amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining

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whether an agent is capable of inhibiting HIV-1 infection
comprising steps of: (a) fixing a chemokine receptor on a
solid matrix; (b) contacting the agent with the fixed
chemokine receptor; (c) contacting the mixture in step (b)
5   with a gp120 in the presence of CD4 under conditions
permitting the binding of the gp120/CD4 complex and the
chemokine receptor in the absence of the agent; (d)
measuring the amount of bound gp120/CD4 complex; and (e)
comparing the amount determined in step (d) with the amount
10   determined in the absence of the agent, a decrease of the
amount indicating that the agent is capable of inhibiting
HIV-1 infection.

This invention also provides a method for determining
15   whether an agent is capable of inhibiting HIV-1 infection
comprising steps of: (a) contacting the agent with a
gp120/CD4 complex under conditions permitting the binding of
the agent to the gp120/CD4 complex; (b) contacting the
gp120/CD4 complex resulting from step (a) with a chemokine
20   receptor under conditions permitting the binding of the
gp120/CD4 complex and the chemokine receptor in the absence
of the agent; (c) measuring the amount of bound chemokine
receptor, wherein a decrease of the amount when compared
with the amount determined in the absence of the agent
25   indicates that the agent is capable of inhibiting HIV-1
infection.

This invention also provides a method for determining
whether an agent is capable of inhibiting HIV-1 infection
30   comprising steps of: (a) fixing a gp120/CD4 complex on a
solid matrix; (b) contacting the agent with the fixed
gp120/CD4 complex under conditions permitting the binding of
the agent to the gp120/CD4 complex; (c) removing unbound
agent;(d) contacting the fixed gp120/CD4 complex resulting
35   from step (c) with a chemokine receptor under conditions
permitting the binding of the gp120/CD4 complex and the
chemokine receptor in the absence of the agent; (e)
measuring the amount of bound chemokine receptor; and (f)

comparing the amount determined in step (e) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

5

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a gp120/CD4 on a solid matrix; (b) contacting the agent with the fixed gp120/CD4 complex; (c) 10 contacting the mixture in step (b) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (d) measuring the amount of bound chemokine receptor; (e) comparing the amount determined in step (d) with the 15 amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

As used in these assays, CD4 include soluble CD4, fragments 20 of CD4 or polypeptides incorporating the gp120 binding site of CD4 capable of binding gp120 and enabling the binding of gp120 to the appropriate chemokine receptor.

As used in these assay gp120 is the gp120 from an 25 appropriate strain of HIV-1. For example, gp120 from the macrophage tropic clinical isolate HIV-1$_{JR-FL}$ will bind to the chemokine receptor CCR5, whereas gp120 from the laboratory adapted T-tropic strain HIV-1$_{LAI}$ will bind to the chemokine receptor CXCR4.

30

In a preferred embodiment of the above methods, the CD4 is a soluble CD4. The chemokine receptor which may be used in the above assay includes CCR5, CXCR4, CCR3 and CCR-2b.

35 In an embodiment, the chemokine receptor is expressed on a cell. In another embodiment, the chemokine receptor is embedded in liposomes. In further embodiment, the chemokine receptor is embedded in a membrane derived from cells

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expressing the chemokine receptor. In a preferred
embodiment, the cell is a L1.2 cell. In a separate
embodiment, the chemokine receptor is purified and
reconstituted in liposomes. Such chemokine receptor
5   embedded in the lipid bilayer of liposomes retains the gp120
binding activity of the receptor.

The gp120, CD4 or both may be labelled with a detectable
marker in the above assays. Markers including radioisotope
10   or enzymes such as horse radish peroxidase may be used in
this invention.

In an embodiment, the gp120 or CD4 or the chemokine receptor
is labelled with biotin. In a further embodiment, the
15   biotinylated gp120, or CD4 or the chemokine receptor is
detected by: (i) incubating with streptavidin-phycoerythrin,
(ii) washing the incubated mixture resulting from step (i),
and (iii) measuring the amount of bound gp120 using a plate
reader, exciting at 530nm, reading emission at 590nm.
20

This invention also provides an agent determined to be
capable of inhibiting HIV-1 infection by the above methods,
which is previously unknown.

25   This invention also provides a pharmaceutical composition
comprising the agent determined to be capable of inhibiting
HIV-1 infection by the above methods and a pharmaceutically
acceptable carrier. In an embodiment, the agent is an
oligopeptide. In another embodiment, the agent is a
30   polypeptide. In a still another embodiment, the agent is a
nonpeptidyl agent.

This invention also provides a molecule capable of binding
to the chemokine receptor CCR5 and inhibiting fusion of HIV-
35   1 to CD4$^+$ cells comprising the above determined agent linked
to a compound capable of increasing the *in vivo* half-life of
the non-chemokine agent. In an embodiment, the compound is
polyethylene glycol. This invention also provides a

pharmaceutical composition comprising an amount of the above molecule effective to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

5   This invention provides a method for reducing the likelihood of HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for treating HIV-1
10   infection in a subject comprising administering the above pharmaceutical composition to the subject.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the
15   art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

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Experimental Details

FIRST SERIES OF EXPERIMENTS

1)   Chemokines inhibit fusion mediated by the envelope glycoprotein from a macrophage-tropic primary isolate of HIV-1 but not from a laboratory-adapted T-lymphotrophic strain of the virus

The chemokines RANTES, MIP-1α and MIP-1β were obtained from R & D systems (Minneapolis, MN). They were tested in the RET assay for ability to inhibit fusion between HeLa-env$_{JR-FL}$ cells (expressing gp120/gp41 from the macrophage tropic isolate HIV-1$_{JR-FL}$) and PM1 cells, or for inhibition of fusion between HeLa-env$_{LAI}$ cells (expressing gp120/gp41 from the laboratory-adapted strain HIV-1$_{LAI}$) and various CD4$^+$ T lymphocyte cell lines. As shown in Figure 1, all three chemokines inhibited fusion mediated by the macrophage tropic virus envelope glycoprotein, but not that mediated by the laboratory-adapted strain envelope glycoprotein.

The ability of the chemokines to block the interaction between CD4 and HIV-1 gp120 which occurs at virus attachment was then tested. It was found that the chemokines did not inhibit this interaction (Figure 2), demonstrating that their blockade of HIV-1 envelope glycoprotein-mediated membrane fusion occurs at the membrane fusion event itself, rather than the initial CD4-gp120 interaction which precedes fusion.

2)   Non-chemokine peptides and derivatives that inhibit HIV-1 fusion

The non-chemokines include chemokine fragments and chemokine derivatives that are tested in the RET assay to determine which are active in inhibiting HIV-1 membrane fusion. Particular attention is focused on fragments or derivatives that inhibit HIV-1 fusion but do not activate leukocyte responses. These non-chemokines include:

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a) N-terminal derivatives of the chemokines. Addition of residues to the N-terminus of chemokines inhibits the function of these proteins without significantly reducing their ability to bind chemokine receptors. For example, Met-RANTES (RANTES with an N-terminal methionine) has been shown to be a powerful antagonist of native RANTES and is unable to induce chemotaxis or calcium mobilization in certain systems. The mechanism of antagonism appears to be competition for receptor binding (9). Similar results were found using other derivatives of the N terminus of RANTES(9) and also by N-terminal modification of other chemokines, such as IL-8 (a member of the C-X-C chemokines) (10). The current invention includes Met-RANTES and other chemokines derivatised by the addition of methionine, or other residues, to the N-terminus so that they inhibit fusion mediated by the envelope glycoprotein of HIV-1$_{JR-FL}$, and inhibit infection by many isolates of HIV-1, yet do not activate the inflammatory response.

b) Chemokines with N-terminal amino acids deleted: Chemokine antagonists have been generated by deleting amino acids in the N-terminal region. For example, deletion of up to 8 amino acids at the N-terminus of the chemokine MCP-1 (a member of the C-C chemokine group), ablated the bioactivity of the protein while allowing it to retain chemokine receptor binding and the ability to inhibit activity of native MCP-1 (11,12).

The current invention includes N-terminal deletants of RANTES, MIP-1α and MIP-1β, lacking the biological activity of the native proteins, which inhibit HIV-1 fusion and HIV-1 infection.

c) Other peptides: A series of overlapping peptides (e.g. of 20-67 residues) from all regions of RANTES, MIP-1α and MIP-1β are screened by the same approaches to identify peptides which inhibit HIV-1 fusion most potently without activating leukocytes. Activation of leukocyte responses is

measured following routine procedures (9, 10, 11, 12).

3)   Cloning the chemokine receptors

Chemokine receptors required for HIV-1 fusion are cloned by the following strategy. First a cDNA library is made in a mammalian expression vector (e.g. pcDNA3.1 from Invitrogen Corp. San Diego, CA) using mRNA prepared from the PM1 cell line or CD4⁺ T-lymphocytes or macrophages. Degenerate oligonucleotide probes are used to identify members of the cDNA library encoding members of the chemokine receptor family, for example following previously published methods (2). The vectors containing chemokine receptor cDNAs are then individually expressed in one of several mammalian cell lines which express human CD4 but do not fuse with HeLa-env$_{JR-FL}$ cells (e.g. HeLa-CD4, CHO-CD4 or COS-CD4) or HeLa-env$_{LAI}$ cells (e.g. CHO-CD4 or COS-CD4). Following analysis in the RET assay, clones which gain the ability to fuse with HeLa-env$_{JR-FL}$ or HeLa-env$_{LAI}$ are identified and the coding sequences recovered, for example by PCR amplification, following procedures well known to those skilled in the art. DNA sequencing is then performed to determine whether the cDNA recovered encodes a known chemokine receptor. Following expression of the receptor, monoclonal and polyclonal antibodies are prepared and tested for ability to inhibit infection by a panel of HIV-1 isolates.

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SECOND SERIES OF EXPERIMENTS

The replication of primary, non-syncytium-inducing (NSI)
HIV-1 isolates in CD4$^+$ T-cells is inhibited by the C-C
ß-chemokines MIP-1α, MIP-1ß and RANTES (1,2), but T-cell
5   line-adapted (TCLA) or syncytium-inducing (SI) primary
strains are insensitive (2,3). The ß-chemokines are small
(8kDa), related proteins active on cells of the lymphoid and
monocyte lineage (4-8). Their receptors are members of the
7-membrane-spanning, G-protein-linked superfamily, one of
10   which (the LESTR orphan receptor) has been identified as the
second receptor for TCLA HIV-1 strains, and is now
designated fusin (9). Fusin is not known to be a
ß-chemokine receptor (7-9).

15   To study how ß-chemokines inhibit HIV-1 replication, a virus
entry assay based on single-cycle infection by an
env-deficient virus, NL4/3Δenv (which also carries the
luciferase reporter gene), complemented by envelope
glycoproteins expressed in trans was used (10,11). Various
20   env-complemented viruses were tested in PM1 cells, a variant
of HUT-78 that has the unique ability to support replication
of primary and TCLA HIV-1 strains, allowing comparison of
envelope glycoprotein functions against a common cellular
background (2,12). MIP-1α, MIP-1ß and RANTES are most active
25   against HIV-1 in combination (2,3), and strongly inhibited
infection of PM1 cells by complemented viruses whose
envelopes are derived from the NSI primary strains ADA and
BaL (Table 1a).

Table 1: Inhibition of HIV-1 entry in PM1 cells and CD4⁺ T-cells by ß-chemokines

% luciferase activity

a)	BaL	ADA	NL4/3	HxB2	MuLV
PM1 cells					
control without virus	2	2	2	5	3
control with virus	100	100	100	100	100
+R/Mα/Mß (50/50/50)	2	3	92	117	100
+RANTES (100)	1	1	nd	nd	nd
+MIP-1α(100)	54	54	nd	nd	nd
+MIP-1ß (100)	1	6	nd	nd	nd
+MCP-1 (100)	46	50	nd	nd	nd
+MCP-2 (100)	28	26	nd	nd	nd
+MCP-3 (100)	58	46	nd	nd	nd
b)	JR-FL	HxB2	MuLV		
LW4 CD4⁺ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	14	68	nd		
LW5 CD4⁺ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	15	73	nd		

Table 1 legend:

PM1 cells were cultured as described by Lusso et al (12).
Ficoll/hypaque-isolated PBMC from laboratory workers (LW)
stimulated with PHA for 72h before depletion of CD8+
Lymphocytes with anti-CD8 immunomagnetic beads (DYNAL,
Great Neck, NY). CD4+ Lymphocytes were maintained in
culture medium containing interleukin-2 (100U/ml; Hofmann
LaRoche, Nutley, NJ), as described previously (3).
Target cells (1-2x10⁵) were infected with supernatants
(10-50ng of HIV-1 p24) from 293-cells co-transfected with
an NL4/3Δenv-luciferase vector and a HIV-1 env-expressing
vector (10,11). ß-Chemokines (R & D Systems,
Minneapolis) were added to the target cells
simultaneously with virus, at the final concentrations
(ng/ml) indicated in parentheses in the first column. The
ß-chemokine concentration range was selected based on
prior studies (2,3). After 2h, the cells were washed
twice with PBS, resuspended in ß-chemokine-containing
media and maintained for 48-96h. Luciferase activity in

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cell lysates was measured as described previously (10,11). The values indicated represent luciferase activity (cpm)/ng p24/mg protein, expressed relative to that in virus-control cultures lacking ß-chemokines (100%), and are the means of duplicate or sextuplicate determinations. nd, not done. R/Mα/Mß, RANTES + MIP-1α + MIP-1ß.

RANTES and MIP-1ß were strongly active when added individually, while other ß-chemokines - MlP-1α, MCP-1, MCP-2 and MCP-3 (refs. 13-15) - were weaker inhibitors (Table 1a). However, MIP-1α, MIP-1ß and RANTES, in combination, did not inhibit infection of PM1 cells by the TCLA strains NL4/3 and HxB2, or by the amphotropic murine leukemia virus (MuLV-Ampho) pseudotype (Table 1a). Thus, phenotypic characteristics of the HIV-1 envelope glycoproteins influence their sensitivity to ß-chemokines in a virus entry assay.

The env-complementation assay was used to assess HIV-1 entry into CD4+ T-cells from two control individuals (LW4 and LW5). MIP-1α, MIP-1ß and RANTES strongly inhibited infection by the NSI primary strain JR-FL infection of LW4's and LW5's CD4$^+$ T-cells, and weakly reduced HxB2 infection of LW cells (Table 1b), suggesting that there may be some overlap in receptor usage on activated CD4$^+$ T-cells by different virus strains. BaL env-mediated replication in normal PBL was also inhibited by MIP-1α, MIP-1ß and RANTES, albeit with significant inter-donor variation in sensitivity (data not shown).

It was determined when ß-chemokines inhibited HIV-1 replication by showing that complete inhibition of infection of PM1 cells required the continuous presence of ß-chemokines for up to 5h after addition of ADA or BaL env-complemented virus (Fig.3a). Pre-treatment of the cells with ß-chemokines for 2h or 24h prior to infection had no inhibitory effect if the cells were subsequently

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washed before virus addition. Furthermore, adding
ß-chemokines 2h after virus only minimally affected virus
entry (Fig.3a). A PCR-based assay was next used to detect
HIV-1 early DNA reverse transcripts in PM1 cells after
5   10h of infection; reverse transcription of ADA, but not
of NL4/3, could not be detected in the presence of MIP-1ß
and RANTES (Fig.3b). Thus, inhibition by ß-chemokines
requires their presence during at least one of the early
stages of HIV-1 replication: virus attachment, fusion and
10   early reverse transcription.

As described in part in the First Series of Experiments,
these sites of action were discriminated, first by
testing whether ß-chemokines inhibited binding of JR-FL
15   or BRU (LAI) gp120 to soluble CD4, or of tetrameric
CD4-IgG2 binding to HeLa-JR-FL cells expressing
oligomeric envelope glycoproteins (17). No inhibition by
any of the ß-chemokines was found in either assay,
whereas the OKT4a CD4-MAb was strongly inhibitory in both
20   (Fig. 2 and data not shown). Thus, ß-chemokines inhibit
a step after CD4 binding, when conformational changes in
the envelope glycoproteins lead to fusion of the viral
and cellular membranes (18). Cell-cell membrane fusion is
also induced by the gp120-CD4 interaction, and can be
25   monitored directly by resonance energy transfer (RET)
between fluorescent dyes incorporated into cell membranes
(17). In the RET assay, OKT4a completely inhibits
membrane fusion of PM1 cells with HeLa cells expressing
the envelope glycoproteins of either JR-FL (HeLa-JR-FL,
30   the same cell line referred to above as HeLa-env$_{JR-FL}$) or
BRU (HeLa-BRU, the same cell line referred to above as
HeLa-env$_{LAI}$), confirming the specificity of the process
(17). RANTES, MIP-1ß (and to a lesser extent, MIP-1α)
strongly inhibited membrane fusion of HeLa-JR-FL cells
35   with PM1 cells, whereas fusion between PM1 cells and
HeLa-BRU cells was insensitive to these ß-chemokines
(Fig. 1 and Table 2a).

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Table 2: Effect of ß-chemokines on HIV-1 envelope glycoprotein-mediated membrane fusion measured using the RET assay

	% Fusion	
	HeLa-JR-FL	HeLa-BRU
a) <u>PM1 cells</u> no chemokines +R/Mα/Mß (80/400/100) +RANTES (80) +MIP-1α (400) +MIP-1ß (100) +MCP-1 (100) +MCP-2 (100) +MCP-3 (100)	100 1 8 39 13 99 72 98	100 95 100 100 93 98 93 99
b) <u>LW5 CD4+ cells</u> no chemokines +R/Mα/Mß(106/533/133) +RANTES (106) +MIP-1α (533) +MIP-1ß (133) +OKT4A (3ug/ml)	100 39 65 72 44 0	100 100 95 100 92 0

Table 2 legend:

10   *CD4+ target cells (mitogen-activated CD4+ lymphocytes or PM1 cells) were labeled with octadecyl rhodamine (Molecular Probes, Eugene, OR), and HeLa-JR-FL cells, HeLa-BRU cells (or control HeLa cells, not shown) were labeled with octadecyl fluorescein (Molecular Probes), overnight at 37°C. Equal numbers of labeled target cells*

15   *and env-expressing cells were mixed in 96-well plates and ß-chemokines (or CD4 MAb OKT4a) were added at the final concentrations (ng/ml) indicated in parentheses in the first column. Fluorescence emission values were determined 4h after cell mixing (17). If cell fusion occurs, the dyes are closely associated in the conjoined membrane such that excitation of fluorescein at 450nm results in resonance energy transfer (RET) and emission*

5   *by rhodamine at 590nm. Percentage fusion is defined as equal to 100 x [(Exp RET - Min RET) / (Max RET - Min RET)], where Max RET = %RET obtained when HeLa-Env and CD4+ cells are mixed, Exp RET = %RET obtained when HeLa-Env and CD4+ cells are mixed in the presence of*

10   *fusion-inhibitory compounds, and Min RET = %RET obtained*

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when HeLa cells (lacking HIV-1 envelope glycoproteins)
and CD4⁺ cells are mixed. The %RET value is defined by
a calculation described elsewhere(17), and each is the
mean of triplicate determinations. These values were,
5   *for HeLa-JR-FL and HeLa-BRU cells respectively: PM1 cells*
11.5%, 10.5%; LW5 CD4⁺ cells, 6.0%, 10.5%; R/Mα/Mß, RANTES
+ MIP-1α + MIP-1ß.

Similar results were obtained with primary CD4⁺ T-cells
10   from LW5 (Table 2b), although higher concentrations of
ß-chemokines were required to inhibit membrane fusion in
the primary cells than in PM1 cells. Thus, the actions of
the ß-chemokines are not restricted to the PM1 cell line.
The RET assay demonstrates that ß-chemokines interfere
15   with env-mediated membrane fusion.

The simplest explanation of these results is that the
binding of certain ß-chemokines to their receptor(s)
prevents, directly or otherwise, the fusion of HIV-1 with
20   CD4⁺ T-cells. It has been known for a decade that HIV-1
requires a second receptor for entry into CD4⁺ cells
(19-21). This function is supplied, for TCLA strains, by
fusin (9). Several receptors for MIP-1α, MIP-1ß and
RANTES have been identified (6,7), and ß-chemokines
25   exhibit considerable cross-reactivity in receptor usage
(4-8). However, C-C CKR-1 and, especially, C-C CKR-5
were identified as the most likely candidates, based on
tissue expression patterns and their abilities to bind
MIP-1α, MIP-1ß and RANTES (4,7,8,15,22). C-C CKR-1, C-C
30   CKR-5 and LESTR are each expressed at the mRNA level in
PM1 cells and primary macrophages (data not shown).
These and other ß-chemokine receptors were therefore
PCR-amplified, cloned and expressed.

35   The expression of C-C CKR-5 in HeLa-CD4 (human), COS-CD4
(simian) and 3T3-CD4 (murine) cells rendered each of them
readily infectible by the primary, NSI strains ADA and
BaL in the env-complementation assay of HIV-1 entry
(Table 3).

Table 3: C-C CKR-5 expression permits infection of CD4-expressing cells by primary, NSI HIV-1 strains

		pcDNA3.1	LESTR	CKR-1	CKR-2a
COS-CD4	ADA	798	456	600	816
	BaL	660	378	600	636
	HxB2	5800	96700	5240	5070
HeLa-CD4	ADA	678	558	4500	912
	BaL	630	738	1800	654
	HxB2	337000	nd	nd	nd
3T3-CD4	ADA	468	558	450	618
	BaL	606	738	660	738
	HxB2	456	24800	618	672

					R/Mα/Mβ
		CKR-3	CKR-4	CKR-5	CKR-5
COS-CD4	ADA	516	534	153000	3210
	BaL	516	618	58800	756
	HxB2	5470	5620	4850	5000
HeLa-CD4	ADA	558	600	310000	6336
	BaL	516	636	104000	750
	HxB2	nd	nd	nd	356000
3T3-CD4	ADA	534	606	28400	1220
	BaL	534	558	11700	756
	HxB2	732	606	618	606

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Table 3 legend:

Chemokine receptor genes C-C CKR-1, C-C CKR-2a, C-C CKR-3, C-C CKR-4 and C-C CKR-5 have no introns
5 *(4-8,15,22) and were isolated by PCR performed directly on a human genomic DNA pool derived from the PBMC of seven healthy donors. Oligonucleotides overlapping the ATG and the stop codons and containing BamHI and XhoI restriction sites for directional cloning into the*
10 *pcDNA3.1 expression vector (Invitrogen Inc.) were used. LESTR (also known as fusin or HUMSTR) (4,9,24) was cloned by PCR performed directly on cDNA derived from PM1 cells, using sequences derived from the NIH database. Listed below are the 5' and 3' primer pairs used in first (5-1*
15 *and 3-1) and second (5-2 and 3-2) round PCR amplification of the CKR genes directly from human genomic DNA, and of LESTR from PM1 cDNA. Only a single set of primers was used to amplify CKR-5.*

LESTR: L/5-1 = AAG CTT GGA GAA CCA GCG GTT ACC ATG GAG
20 *GGG ATC (SEQ ID NO: 6);*

L/5-2 = GTC TGA GTC TGA GTC AAG CTT GGA GAA CCA (SEQ ID NO: 7);

L/3-1 = CTC GAG CAT CTG TGT TAG CTG GAG TGA AAA CTT GAA GAC TC (SEQ ID NO: 8);

25 *L/3-2 = GTC TGA GTC TGA GTC CTC GAG CAT CTG TGT (SEQ ID NO: 9);*

CKR-1:C1/5-1 = AAG CTT CAG AGA GAA GCC GGG ATG GAA ACT CC (SEQ ID NO: 10);

C1/5-2 = GTC TGA GTC TGA GTC AAG CTT CAG AGA GAA (SEQ ID
30 *NO: 11);*

C1/3-1 = CTC GAG CTG AGT CAG AAC CCA GCA GAG AGT TC (SEQ ID NO: 12);

C1/3-2 = GTC TGA GTC TGA GTC CTC GAG CTG AGT CAG (SEQ ID NO: 13);

35 *CKR-2a:C2/5-1 = AAG CTT CAG TAC ATC CAC AAC ATG CTG TCC AC (SEQ ID NO: 14);*

C2/5-2= GTC TGA GTC TGA GTC AAG CTT CAG TAC ATC (SEQ ID NO: 15);

C2/3-1 = CTC GAG CCT CGT TTT ATA AAC CAG CCG AGA C (SEQ

ID NO: 16);

C2/3-2 = GTC TGA GTC TGA GTC CTC GAG CCT CGT TTT (SEQ ID
NO: 17);

CKR-3: C3/5-1 = AAG CTT CAG GGA GAA GTG AAA TGA CAA CC
(SEQ ID NO: 18);

C3/5-2= GTC TGA GTC TGA GTC AAG CTT CAG GGA GAA (SEQ ID
NO: 19);

C3/3-1 = CTC GAG CAG ACC TAA AAC ACA ATA GAG AGT TCC (SEQ
ID NO: 20);

C3/3-2 = GTC TGA GTC TGA GTC CTC GAG CAG ACC TAA (SEQ ID
NO: 21);

CKR-4: C4/5-1 = AAG CTT CTG TAG AGT TAA AAA ATG AAC CCC
ACG G (SEQ ID NO: 22);

C4/5-2 = GTC TGA GTC TGA GTC AAG CTT CTG TAG AGT (SEQ ID
NO: 23);

C4/3-1 = CTC GAG CCA TTT CAT TTT TCT ACA GGA CAG CAT C
(SEQ ID NO: 24);

C4/3-2 = GTC TGA GTC TGA GTC CTC GAG CCA TTT CAT (SEQ ID
NO: 25);

CKR-5: C5/5-12 = GTC TGA GTC TGA GTC AAG CTT AAC AAG ATG
GAT TAT CAA (SEQ ID NO: 26);

C5/3-12 = GTC TGA GTC TGA GTC CTC GAG TCC GTG TCA CAA GCC
CAC (SEQ ID NO: 37).

The human CD4-expressing cell lines HeLa-CD4 (P42),
3T3-CD4 (sc6) and COS-CD4 (Z28T1) (23) were transfected
with the different pcDNA3.1-CKR constructs by the calcium
phosphate method, then infected 48h later with different
reporter viruses (200ng of HIV-1 $p24/10^6$ cells) in the
presence or absence of ß-chemokines (400ng/ml each of
RANTES, MIP-1α and MIP-1ß). Luciferase activity in cell
lysates was measured 48h later (10,11). ß-Chemokine
blocking data is only shown for C-C CKR-5, as infection
mediated by the other C-C CKR genes was too weak for
inhibition to be quantifiable. In PCR-based assays of
HIV-1 entry, a low level of entry of NL4/3 and ADA into
C-C CKR-1 expressing cells (data not shown) was
consistently observed.

Neither LESTR nor C-C CKR-1, -2a, -3 or -4 could

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substitute for C-C CKR-5 in this assay. The expression of
LESTR in COS-CD4 and 3T3-CD4 cells permitted HxB2 entry,
and HxB2 readily entered untransfected (or control
plasmid-transfected) HeLa-CD4 cells (Table 3). Entry of
5   BAL and ADA into all three C-C CKR-5-expressing cell
lines was almost completely inhibited by the combination
of MIP-1α, MIP-1ß and RANTES, whereas HxB2 entry into
LESTR-expressing cells was insensitive to ß chemokines
(Table 3). These results suggest that C-C CKR-5 functions
10   as a ß-chemokine-sensitive second receptor for primary,
NSI HIV-1 strains.

The second receptor function of C-C CKR-5 was confirmed
in assays of env-mediated membrane fusion. When C-C CKR-5
15   was transiently expressed in COS and HeLa cell lines that
permanently expressed human CD4, both cell lines fused
strongly with HeLa cells expressing the JR-FL envelope
glycoproteins, whereas no fusion occurred when control
plasmids were used (data not shown). Expression of LESTR
20   instead of C-C CKR-5 did not permit either COS-CD4 or
HeLa-CD4 cells to fuse with HeLa-JR-FL cells, but did
allow fusion between COS-CD4 cells and HeLa-BRU cells
(data not shown).

25   The fusion capacity of ß-chemokine receptors was also
tested in the RET assay. The expression of C-C CKR-5,
but not of C-C CKR-1, -2a, -3 or -4, permitted strong
fusion between HeLa-CD4 cells and HeLa-JR-FL cells. The
extent of fusion between HeLa-JR-FL cells and C-C
30   CKR-5-expressing HeLa-CD4 cells was greater than the
constitutive level of fusion between HeLa-BRU cells and
HeLa-CD4 cells (Fig.4). The fusion-conferring function
of C-C CKR-5 for primary, NSI HIV-1 strains has therefore
been confirmed in two independent fusion assays.

35

Experimental Discussion

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Together, the above results establish that MlP-1α, MIP-1ß and RANTES inhibit HIV-1 infection at the entry stage, by interfering with the virus-cell fusion reaction subsequent to CD4 binding. It was also shown that C-C CKR-5 can serve as a second receptor for entry of primary NSI strains of HIV-1 into CD4+ T-cells, and that the interaction of ß-chemokines with C-C CKR-5 inhibits the HIV-1 fusion reaction.

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References of the Second Series of Experiments

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THIRD SERIES OF EXPERIMENTS

The chemokine SDF-1 (stromal cell-derived factor 1) is the natural ligand for Fusin/CXCR4 and blocks infection by laboratory-adapted strains of HIV-1 (Ref. 1 and 2). SDF-1 exists as at least two forms, SDF-1α and SDF-1ß based on variable splicing of the SDF-1 gene (Ref. 1 and 3) In the RET assay, this chemokine specifically inhibits membrane fusion mediated by gp120/gp41 form the laboratory-adapted strain HIV$_{LAI}$ but not by gp120/gp41 from the macrophage-tropic isolate HIV-1$_{JR-FL}$ as shown in Figure 5.

References of the Third Series of Experiments
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FOURTH SERIES OF EXPERIMENTS

Direct Binding of HIV-1$_{JR-FL}$ gp120 to CCR5$^+$ CD4$^-$ Cells

The direct binding of HIV-1$_{JR-FL}$ gp120 to CCR5$^+$ CD4$^-$ cells
has been demonstrated. In this case, preincubation of

5   the gp120 with sCD4 or another CD4-based molecule is
required,   presumably   because   this   results   in   a
conformational change in gp120 that exposes a chemokine
receptor binding site. Figure 6 illustrates the use of
flow   cytometry   to   measure   the   direct   binding   of

10   sCD4/gp120 complexes to human CCR5-bearing murine L1.2
cells. Background levels of binding were observed with
either biotinylated protein alone, or if gp120 from the
laboratory-adapted strain HIV-1$_{LAI}$ is used in place of the
HIV-1$_{JR-FL}$ gp120 (data not shown).

15

This assay has been adapted for drug screening purposes
to a 96-well microplate format where binding of the
sCD4/gp120 complexes to CCR5$^+$/CD4$^-$ cells is measured using
a fluorometric plate reader. One method is as follows:

20

1)   Plate out L1.2-CCR5$^+$ cells (approx. 500,000/well).
2)   Add inhibitor for 1 hour at room temperature.
3)   Wash and add biotinylated sCD4(2.5μg/ml) and
biotinylated HIV-1$_{JR-FL}$ gp120 (5μg/ml), then incubate

25   for 2 hours at room temperature.
4)   Wash and incubate with streptavidin-phycoerythrin
(100ng/nl).
5)   Wash and measure the amount of bound gp120/sCD4
using a fluorometric plate reader exciting at 530nm

30   and reading emission at 590nm.

Using this method, inhibition of binding of gp120/sCD4 to
CCR5 by CC-chemokines (Fig. 7) and antibodies to CCR5
that   block   HIV-1   infection   (not   shown)   have   been

35   demonstrated.

Inhibition of HIV-1 envelope-mediated membrane fusion by
the bicyclam, JM3100.

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The bicyclam JM3100, obtained from Dr. J. Moore (Aaron Diamond AIDS Research Center, NY) was tested for ability to inhibit membrane fusion mediated by the envelope glycoproteins of the LAI or JR-FL strains of HIV-1 using the resonance energy transfer (RET) assay described above. As illustrated in Fig. 7, this molecule specifically and potently inhibits fusion mediated by gp120/gp41 from the HIV-1$_{LAI}$ strain, and not from the HIV-1$_{JR-FL}$ strain. These data suggest that this molecule specifically inhibits HIV fusion by blocking the interaction between HIV-1$_{LAI}$ gp120 and CXCR4.

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FIFTH SERIES OF EXPERIMENTS
CCR5 Receptor Binding Assay

Materials:

1.   CCR5$^+$/L1.2 cell line
2.   L1.2 cell line
3.   JRFL-gp120, biotinylated
4.   sCD4, unconjugated (Intracell, Cat #13101)
5.   96-well round bottom plate (Corning, cat #25850)
6.   Streptavidin, phycoerythrin conjugated [SA-PE] (Becton Dickinson, cat #349023)
7.   PBS without Calcium and Magnesium [PBS(-)] (Gibco BRL, cat #14190)

Method:

1.   Culture CCR5$^+$ and parental L1.2 cells and treat with sodium butyrate as described (Wu et al., J. Exp. Med 185:1681).

2.   Add cells to 96-well plate ($\sim$3x10^5 cells/well)

3.   Centrifuge plate and remove supernatant.

4.   Dilute inhibitory compounds as desired in PBS(-)/0.1% NaN$_3$. Add 40 μl of inhibitory compounds to cells. Add 40 μl of PBS(-)/0.1% NaN$_3$ to wells without inhibitory compounds.

5.   Shake plate to suspend cells in solution. Incubate at room temperature for 1 hour.

6.   Prepare an equimolar ($\sim$50nM) mixture of sCD4 and biotinylated gp120. Add 40μl of sCD4:biotinylated gp120 complex per well. (Final volume in well = 80μl). Shake plate to suspend cells in protein solution. Incubate at room temperature for one hour.

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7. Centrifuge plate and remove supernatant. Add 200μl of PBS(-)0.1% NaN₃ per well. Repeat this washing procedure, for a total of three washes.

5   8. Centrifuge plate and remove supernatant. Dilute SA-PE 1:50 in PBS(-)/0.1% NaN₃ and add 40μl of diluted reagent to cells. Shake plate to suspend cells in solution. Incubate at room temperature for one hour.

10   9. Centrifuge plate as above and remove supernatant. Add 200μl of PBS(-)/0.1% NaN₃ per well. Repeat this washing procedure for a total of three washes.

10. Centrifuge plate as above and remove supernatant. Add
15   200μl of PBS(-)/0.1% NaN₃ per well.

11. Centrifuge plate and measure the fluorescence.
    Emission at 590nm following excitation at 530nm.

20   12. % Inhibition is calculated by using the following formula:

$$\%Inhibition = [Max - Reading] / [Max - Min]$$

       Max = Average of values in wells containing [sCD4:
25   biotinylated gp120 w/CCR5+/L1.2 cells, no inhibitor]

       Min = Average of values in wells containing
       sCD4:biotinylated gp120 w/ L1.2 cells, no inhibitor.

30   Reading = Value in specific well

SEQUENCE LISTING
(1) GENERAL INFORMATION:

        (i) APPLICANTS: Progenics Pharmaceuticals, Inc.

        (ii) TITLE OF INVENTION: A Method For Preventing HIV-1 Infection of
                                 CD4+ Cells

        (iii) NUMBER OF SEQUENCES: 27

        (iv) CORRESPONDENCE ADDRESS:
                (A) ADDRESSEE: Cooper & Dunham LLP
                (B) STREET: 1185 Avenue of the Americas
                (C) CITY: New York
                (D) STATE: New York
                (E) COUNTRY: USA
                (F) ZIP: 10036

        (v) COMPUTER READABLE FORM:
                (A) MEDIUM TYPE: Floppy disk
                (B) COMPUTER: IBM PC compatible
                (C) OPERATING SYSTEM: PC-DOS/MS-DOS
                (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

        (vi) CURRENT APPLICATION DATA:
                (A) APPLICATION NUMBER:
                (B) FILING DATE:
                (C) CLASSIFICATION:

        (viii) ATTORNEY/AGENT INFORMATION:
                (A) NAME: White, John P
                (B) REGISTRATION NUMBER: 28678
                (C) REFERENCE/DOCKET NUMBER: 50875-F-PCT/JPW/AKC

        (ix) TELECOMMUNICATION INFORMATION:
                (A) TELEPHONE: 212-278-0400
                (B) TELEFAX: 212-391-0525

(2) INFORMATION FOR SEQ ID NO:1:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 38 nucleotides
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAGGCTACT TCCCTGATTG GCAGAACTAC ACACCAGG   38

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCAAGCCGA GTCCTGCGTC GAGAG   25

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGACTTTCC GCTGGGGACT TTC
23

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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CCTGTTCGGG CGCCACTGCT AGAGATTTTC CAC   33

(2) INFORMATION FOR SEQ ID NO:5:

5   (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 60 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: n/a
                    (D) TOPOLOGY: n/a
10
               (ii) MOLECULE TYPE: protein

               (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15   Pro Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
             1   5   10   15

            Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe Val
                        20   25   30
20
            Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg
                35   40   45   50

            Glu Tyr Ile Asn Ser Leu Glu Met Ser
25   55   60

            (2) INFORMATION FOR SEQ ID NO:6:
30
               (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 36 nucleotides
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
35   (D) TOPOLOGY: linear

               (ii) MOLECULE TYPE: oligonucleotide

               (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
40
            AAGCTTGGAG AACCAGCGGT TACCATGGAG GGGATC   36

            (2) INFORMATION FOR SEQ ID NO:7:

45   (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 30 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

5   (ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCTGAGTCT GAGTCAAGCT TGGAGAACCA   30

10   (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 41 nucleotides

15   (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

20
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCGAGCATC TGTGTTAGCT GGAGTGAAAA CTTGAAGACT C   41

25   (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 nucleotides
    (B) TYPE: nucleic acid

30   (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

35   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTCTGAGTCT GAGTCCTCGA GCATCTGTGT   30

(2) INFORMATION FOR SEQ ID NO:10:

40
(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 32 nucleotides
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single

45   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGCTTCAGA GAGAAGCCGG GATGGAAACT CC   32

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTCTGAGTCT GAGTCAAGCT TCAGAGAGAA   30

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCGAGCTGA GTCAGAACCC AGCAGAGAGT TC   32

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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GTCTGAGTCT GAGTCCTCGA GCTGAGTCAG   30

(2) INFORMATION FOR SEQ ID NO:14:

5   (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 32 nucleotides
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

10   (ii) MOLECULE TYPE: oligonucleotide

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

15 AAGCTTCAGT ACATCCACAA CATGCTGTCC AC   32

     (2) INFORMATION FOR SEQ ID NO:15:

            (i) SEQUENCE CHARACTERISTICS:
20   (A) LENGTH: 30 nucleotides
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

25   (ii) MOLECULE TYPE: oligonucleotide

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

    GTCTGAGTCT GAGTCAAGCT TCAGTACATC   30
30
     (2) INFORMATION FOR SEQ ID NO:16:

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 31 nucleotides
35   (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: oligonucleotide
40
            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

    CTCGAGCCTC GTTTTATAAA CCAGCCGAGA C   31

45   (2) INFORMATION FOR SEQ ID NO:17:

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```
      (i)  SEQUENCE CHARACTERISTICS:
           (A)  LENGTH: 30 nucleotides
           (B)  TYPE: nucleic acid
           (C)  STRANDEDNESS: single
 5         (D)  TOPOLOGY: linear


      (ii) MOLECULE TYPE: oligonucleotide


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
10
      GTCTGAGTCT GAGTCCTCGA GCCTCGTTTT                          30


      (2)  INFORMATION FOR SEQ ID NO:18:


15         (i)  SEQUENCE CHARACTERISTICS:
               (A)  LENGTH: 29 nucleotides
               (B)  TYPE: nucleic acid
               (C)  STRANDEDNESS: single
               (D)  TOPOLOGY: linear
20
           (ii) MOLECULE TYPE: oligonucleotide


           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:


25    AAGCTTCAGG GAGAAGTGAA ATGACAACC                           29


      (2)  INFORMATION FOR SEQ ID NO:19:


           (i)  SEQUENCE CHARACTERISTICS:
30             (A)  LENGTH: 30 nucleotides
               (B)  TYPE: nucleic acid
               (C)  STRANDEDNESS: single
               (D)  TOPOLOGY: linear


35         (ii) MOLECULE TYPE: oligonucleotide


           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:


      GTCTGAGTCT GAGTCAAGCT TCAGGGAGAA                          30
40
      (2)  INFORMATION FOR SEQ ID NO:20:


           (i)  SEQUENCE CHARACTERISTICS:
               (A)  LENGTH: 33 nucleotides
45             (B)  TYPE: nucleic acid
               (C)  STRANDEDNESS: single
```

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         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: oligonucleotide

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTCGAGCAGA CCTAAAACAC AATAGAGAGT TCC   33

(2) INFORMATION FOR SEQ ID NO:21:

10
         (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 30 nucleotides
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
15   (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: oligonucleotide

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
20
GTCTGAGTCT GAGTCCTCGA GCAGACCTAA   30

(2) INFORMATION FOR SEQ ID NO:22:

25   (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 34 nucleotides
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear
30
         (ii) MOLECULE TYPE: oligonucleotide

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

35   AAGCTTCTGT AGAGTTAAAA AATGAACCCC ACGG   34

(2) INFORMATION FOR SEQ ID NO:23:

         (i) SEQUENCE CHARACTERISTICS:
40   (A) LENGTH: 30 nucleotides
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

45   (ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTCTGAGTCT GAGTCAAGCT TCTGTAGAGT   30

5   (2) INFORMATION FOR SEQ ID NO:24:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 34 nucleotides
             (B) TYPE: nucleic acid
10   (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: oligonucleotide

15   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCGAGCCAT TTCATTTTTC TACAGGACAG CATC   34

    (2) INFORMATION FOR SEQ ID NO:25:
20

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 30 nucleotides
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: oligonucleotide

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
30

GTCTGAGTCT GAGTCCTCGA GCCATTTCAT   30

    (2) INFORMATION FOR SEQ ID NO:26:

35   (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 39 nucleotides
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

40
         (ii) MOLECULE TYPE: oligonucleotide

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

45 GTCTGAGTCT GAGTCAAGCT TAACAAGATG GATTATCAA   39

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 39 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotides

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTCTGAGTCT GAGTCCTCGA GTCCGTGTCA CAAGCCCAC   39

What is claimed is:

1. A non-chemokine agent capable of binding to a chemokine receptor and inhibiting fusion of HIV-1 to CD4⁺ cells with the proviso that the agent is not a known bicyclam or its known derivative.

2. The non-chemokine agent of claim 1, wherein the non-chemokine agent is a oligopeptide.

3. The non-chemokine agent of claim 1, wherein the non-chemokine agent is a nonpeptidyl agent.

4. The non-chemokine agent of claim 1, wherein the non-chemokine agent is a polypeptide.

5. The non-chemokine agent of claim 4, wherein the polypeptide is an antibody or a portion of an antibody.

6. The non-chemokine agent of claim 4, wherein the polypeptide comprises amino acid sequence as set forth in SEQ ID NO:5.

7. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first seven N-terminal amino acids of said sequence.

8. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first eight N-terminal amino acids of said sequence.

9. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first nine N-terminal amino acids of said sequence.

10. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first ten N-terminal amino acids of said sequence.

11. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by addition of an amino acid or oligopeptide.

12. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by removing the N-terminal alanine and replacing it by serine or threonine and an additional amino acid or oligopeptide or nonpeptidyl moiety.

13. The non-chemokine agent of claim 11 or 12, wherein the additional amino acid is methionine.

14. An non-chemokine agent capable of binding to CXCR4 and inhibiting HIV-1 infection with the proviso that the agent is not a known bicyclam or its known derivative.

15. The non-chemokine agent of claim 14, wherein the agent is an oligopeptide.

16. The non-chemokine agent of claim 14, wherein the agent is a polypeptide.

17. The non-chemokine agent of claim 16, wherein the polypeptide comprises the SDF-1 sequence with the deletion of the first six N-terminal amino acids of said sequence.

18. The non-chemokine agent of claim 16, wherein the polypeptide comprises the SDF-1 sequence with the

-61-

deletion of the first seven N-terminal amino acids
of said sequence.

19.   The non-chemokine agent of claim 16, wherein the
      polypeptide comprises the SDF-1 sequence with the
      deletion of the first eight N-terminal amino acids
      of said sequence.

20.   The non-chemokine agent of claim 16, wherein the
      polypeptide comprises the SDF-1 sequence with the
      deletion of the first nine N-terminal amino acids of
      said sequence.

21.   The non-chemokine agent of claim 16, wherein the N-
      terminal glycine of SDF-1 is replaced by serine and
      derivatized with biotin.

22.   The non-chemokine agent of claim 16, wherein the N-
      terminal glycine of SDF-1 is replaced by serine and
      derivatized with methionine.

23.   The non-chemokine agent of claim 16, wherein the N-
      terminus of SDF-1 is modified by the addition of a
      methionine before the terminal glycine.

24.   The agent of claim 16, wherein the agent is an
      antibody or a portion of an antibody.

25.   The agent of claim 14, wherein the agent is a non-
      peptidyl agent.

26.   A pharmaceutical composition comprising an amount of
      the non-chemokine agent of claim 1 effective to
      inhibit fusion of HIV-1 to CD4$^+$ cells and a
      pharmaceutically acceptable carrier.

27.   A pharmaceutical composition comprising an amount of
      the non-chemokine agent of claim 14 effective to

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inhibit fusion of HIV-1 to CD4⁺ cells and a
pharmaceutically acceptable carrier.

28. A composition of matter capable of binding to a
chemokine receptor and inhibiting fusion of HIV-1 to
CD4⁺ cells comprising a non-chemokine agent linked
to a ligand capable of binding to a cell surface
receptor of the CD4⁺ cells other than the chemokine
receptor such that the binding of the non-chemokine
agent to the chemokine receptor does not inhibit the
binding of the ligand to the other receptor.

29. The composition of matter of claim 28, wherein the
cell surface receptor is CD4.

30. The composition of matter of claim 28, wherein the
ligand comprises an antibody or a portion of an
antibody.

31. A pharmaceutical composition comprising an amount of
the composition of matter of claim 28 effective to
inhibit fusion of HIV-1 to CD4⁺ cells and a
pharmaceutically acceptable carrier.

32. A method for reducing the likelihood of HIV-1
infection in a subject comprising administering the
pharmaceutical composition of claim 26, 27, or 31 to
the subject.

33. A method for treating HIV-1 infection in a subject
comprising administering the pharmaceutical
composition of claim 26 or 27 to the subject.

34. A method for determining whether an agent is capable
of inhibiting HIV-1 infection comprising steps of:

    (a) contacting an appropriate concentration of an
        agent with a chemokine receptor or a portion

thereof under conditions permitting the binding
of the agent to the chemokine receptor;

5

(b)   contacting the chemokine receptor resulting
       from step (a) with a gp120/CD4 complex under
       conditions permitting the binding of the
       gp120/CD4 complex and the chemokine receptor in
       the absence of the agent;

10

(c)   measuring the amount of bound gp120/CD4 complex
       wherein a decrease in the amount compared with
       the amount determined in the absence of the
       agent indicates that the agent is capable of
       inhibiting HIV-1 infection.

15

35.   A method for determining whether an agent is capable
       of inhibiting HIV-1 infection comprising steps of:

(a)   fixing a chemokine receptor on a solid matrix;

20

(b)   contacting the agent with the fixed chemokine
       receptor under conditions permitting the
       binding of the agent to the chemokine receptor;

25

(c)   removing the unbound agent;

(d)   contacting the fixed chemokine receptor
       resulting in step (c) with a gp120 in the
       presence of CD4 under conditions permitting the

30

       binding of the gp120/CD4 complex and the
       chemokine receptor in the absence of the agent;

(e)   measuring the amount of bound gp120/CD4
       complex; and

35

(f)   comparing the amount determined in step (d)
       with the amount determined in the absence of
       the agent, a decrease of the amount indicating

that the agent is capable of inhibiting HIV-1 infection.

36. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

    (a)   fixing a chemokine receptor on a solid matrix;

    (b)   contacting the agent with the fixed chemokine receptor;

    (c)   contacting the mixture in step (b) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

    (d)   measuring the amount of bound gp120/CD4 complex; and

    (e)   comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

37. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

    (a)   contacting the agent with a gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex;

    (b)   contacting the gp120/CD4 complex resulting from step (a) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(c) measuring the amount of bound chemokine receptor, wherein a decrease of the amount when compared with the amount determined in the absence of the agent indicates that the agent is capable of inhibiting HIV-1 infection.

38. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) fixing a gp120/CD4 complex on a solid matrix

(b) contacting the agent with the fixed gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex;

(c) removing unbound agent;

(d) contacting the fixed gp120/CD4 complex resulting from step (c) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(e) measuring the amount of bound chemokine receptor; and

(f) comparing the amount determined in step (e) with thte amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

39. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) fixing a gp120/CD4 complex on a solid matrix;

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     (b)   contacting the agent with the fixed gp120/CD4 complex;

     (c)   contacting the mixture in step (b) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

     (d)   measuring the amount of bound chemokine receptor; and

     (e)   comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

40.   The method of claim 34, 35, 36, 37, 38 or 39 wherein the CD4 is a soluble CD4.

41.   The method of claim 34, 35, 36, 37, 38 or 39 wherein the chemokine receptor is CCR5.

42.   The method of claim 34, 35, 36, 37, 38 or 39 wherein the chemokine receptor is CXCR4.

43.   The method of claim 34, 35, 36, 37, 38 or 39 wherein the chemokine receptor is expressed on a cell.

44.   The method of claim 43, wherein the chemokine receptor is embedded in liposomes.

45.   The method of claim 43, wherein the chemokine receptor is embedded in a membrane derived from cells expressing the chemokine receptor.

46.   The method of claim 43, wherein the cell is a L1.2 cell.

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47. The method of claim 35 or 36, wherein the gp120, CD4 or both are labelled with a detectable marker.

48. The method of claim 37, 38 or 39, wherein the chemokine receptor is labelled with a detectable marker.

49. The method of claim 47 or 48, wherein the gp120, CD4 or the chemokine receptor is labelled with biotin.

50. The method of claim 49, wherein the biotinylated gp120, CD4 or the chemokine receptor is detected by:

(i)   incubating   with   streptavidin-phycoerythrin,

(ii)   washing the incubated mixture resulting from step (i), and

(iii)   measuring the amount of bound gp120, CD4 or the chemokine receptor using a fluorometer, exciting at 530nm and reading the emission at 590nm.

51. The agent determined to be capable of inhibiting HIV-1 infection by the method of claim 34, 35, 36, 37, 38 or 39 which is previously unknown.

52. A pharmaceutical composition comprising the agent determined to be capable of inhibiting HIV-1 infection by the method of claim 34, 35, 36, 37, 38 or 39 and a pharmaceutically acceptable carrier.

53. The method of claim 34, 35, 36, 37, 38 or 39 wherein the agent is an oligopeptide.

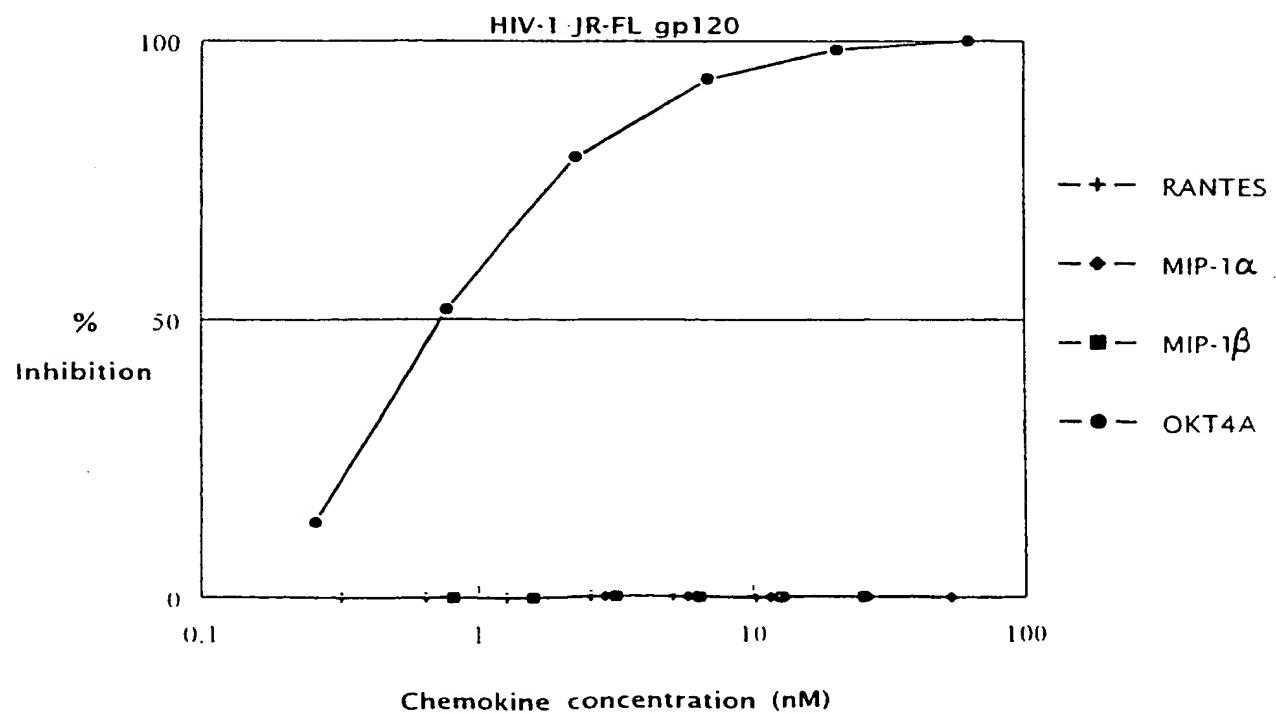54. The method of claim 34, 35, 36, 37, 38 or 39 wherein the agent is a polypeptide.

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55. The method of claim 34, 35, 36, 37, 38 or 39 wherein the agent is a nonpeptidyl agent.

56. The agent of claim 51 linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent.

57. The agent of claim 56, wherein the compound is polyethylene glycol.

58. A pharmaceutical composition comprising an amount of the agent of claim 56 effective to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

59. A method for reducing the likelihood of HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 52 or 58 to the subject.

60. A method for treating HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 52 or 58 to the subject.

FIG. 1A

RANTES

FIG. 1B

MIP-1alpha
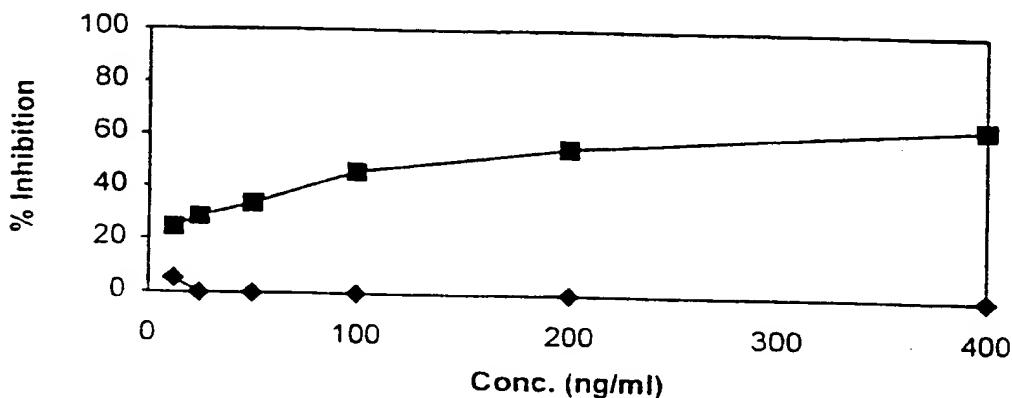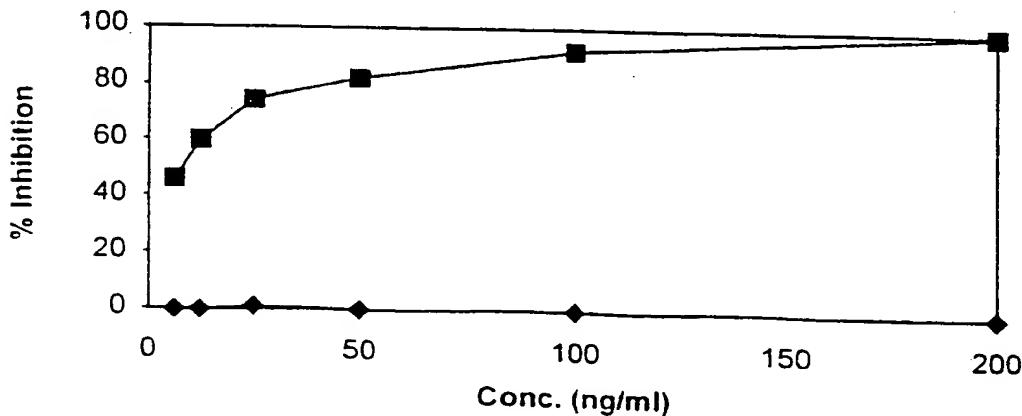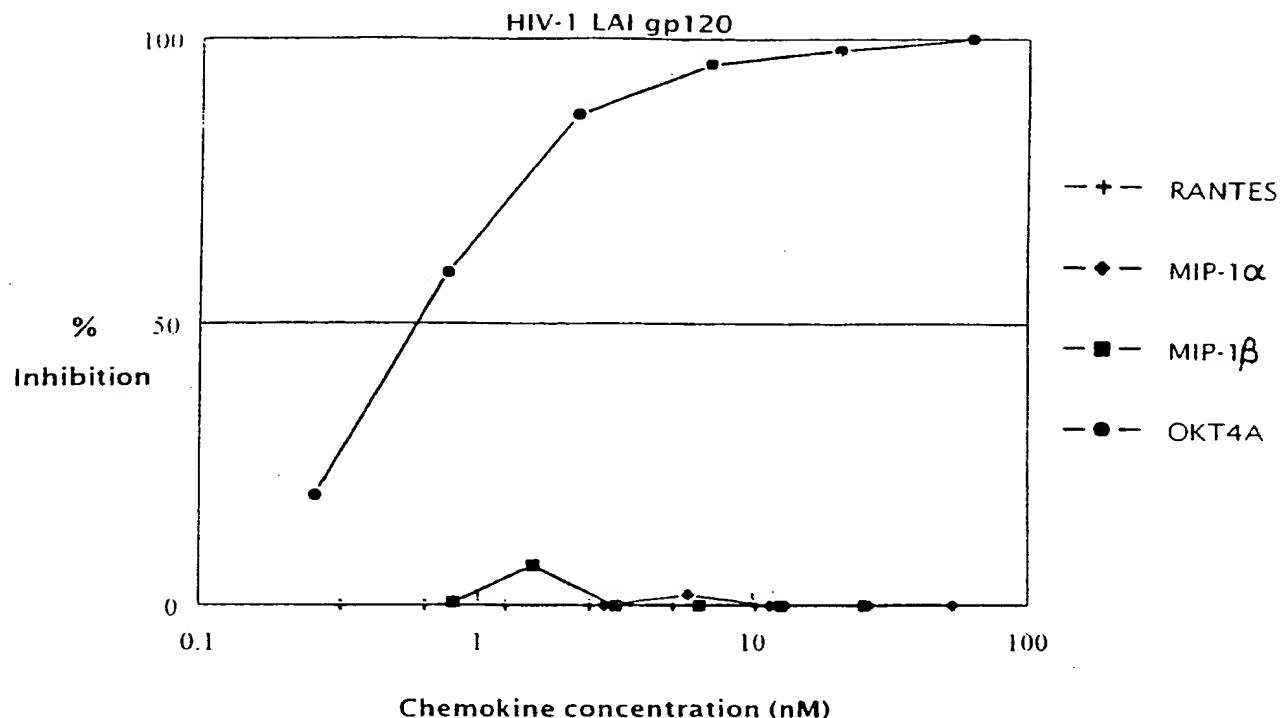
FIG. 1C

MIP-1beta

FIG. 2A

FIG. 2B

FIG. 3A

FIG. 3B

FIG. 4

FIG. 5

FIG. 6B

Streptavidin-PE

Biotinylated gp120 + sCD4

No Stain

60

0

10^0 10^1 10^2 10^3

relative fluorescence units

FIG. 6A

No Stain

Biotinylated gp120 + sCD4

60

0

10^0 10^1 10^2

relative fluorescence units
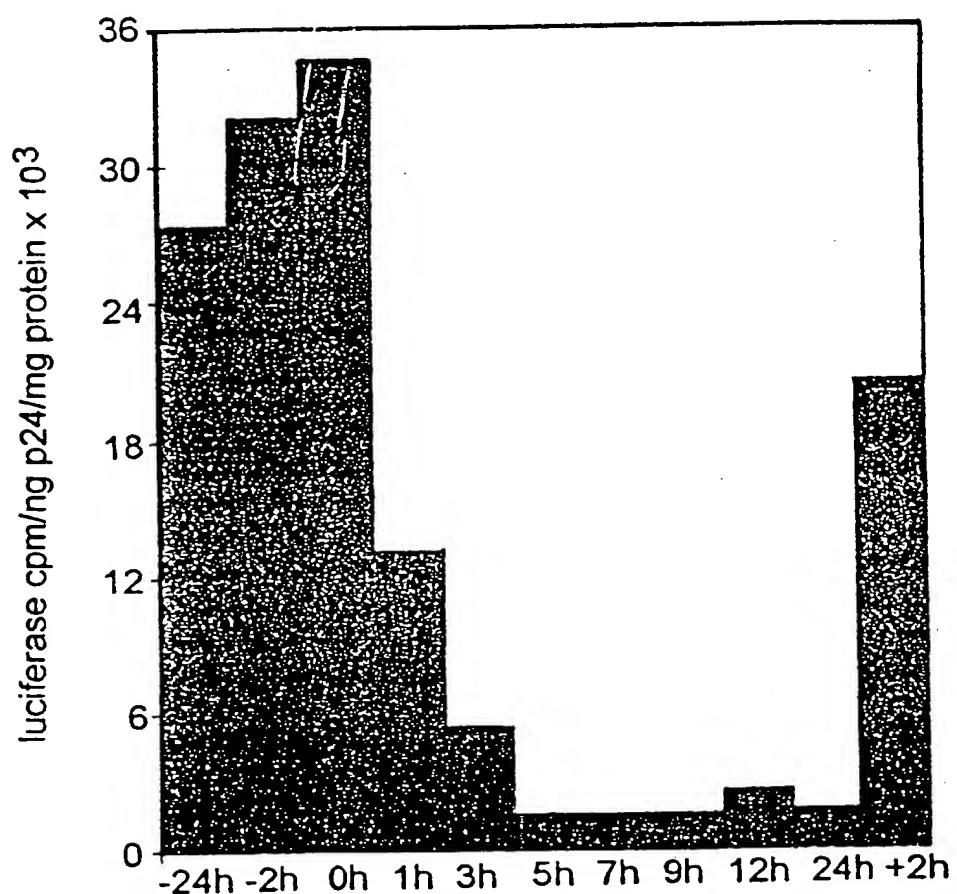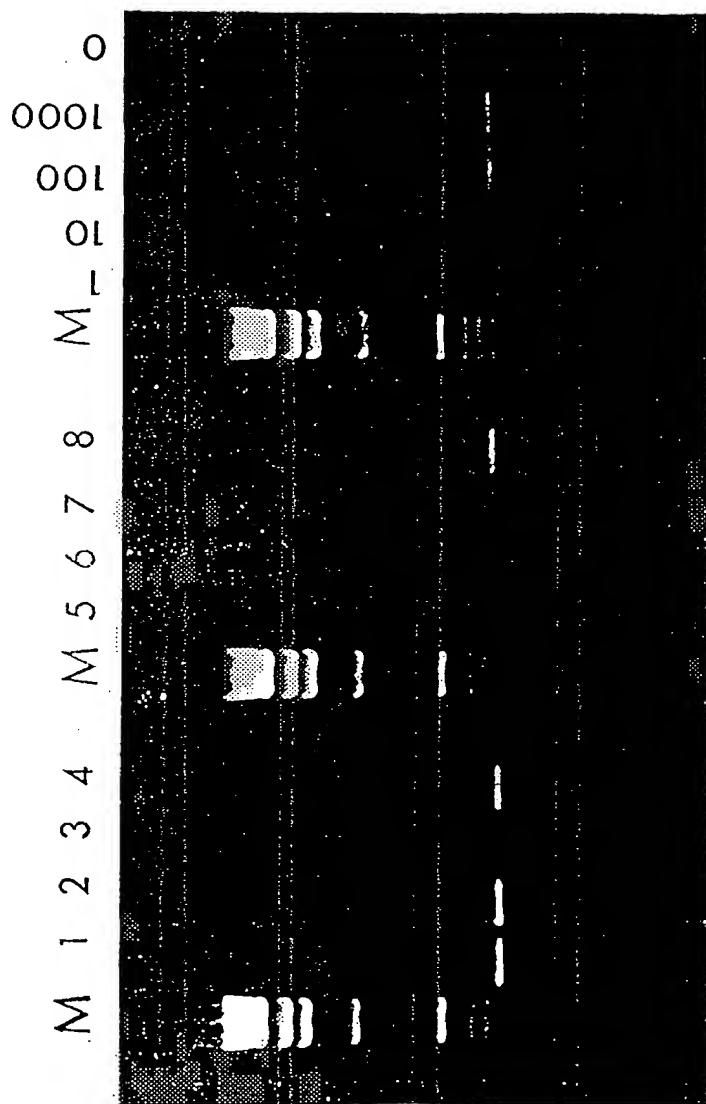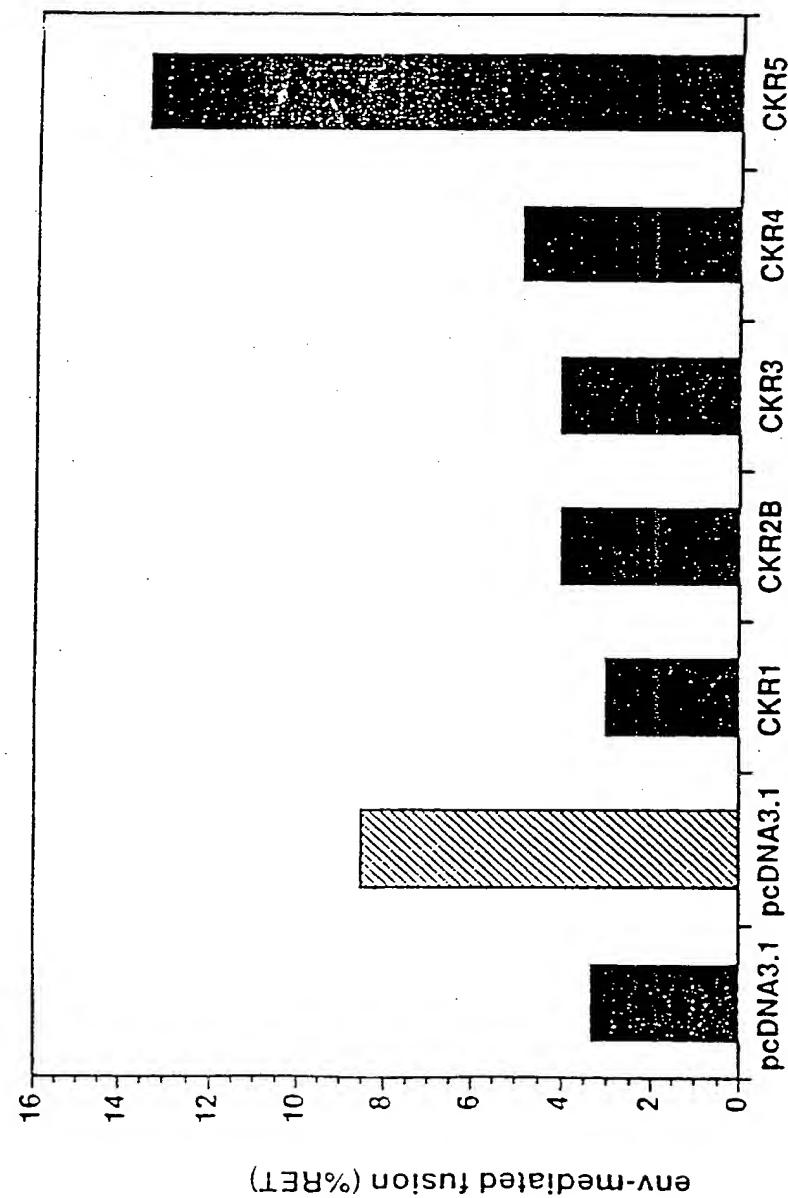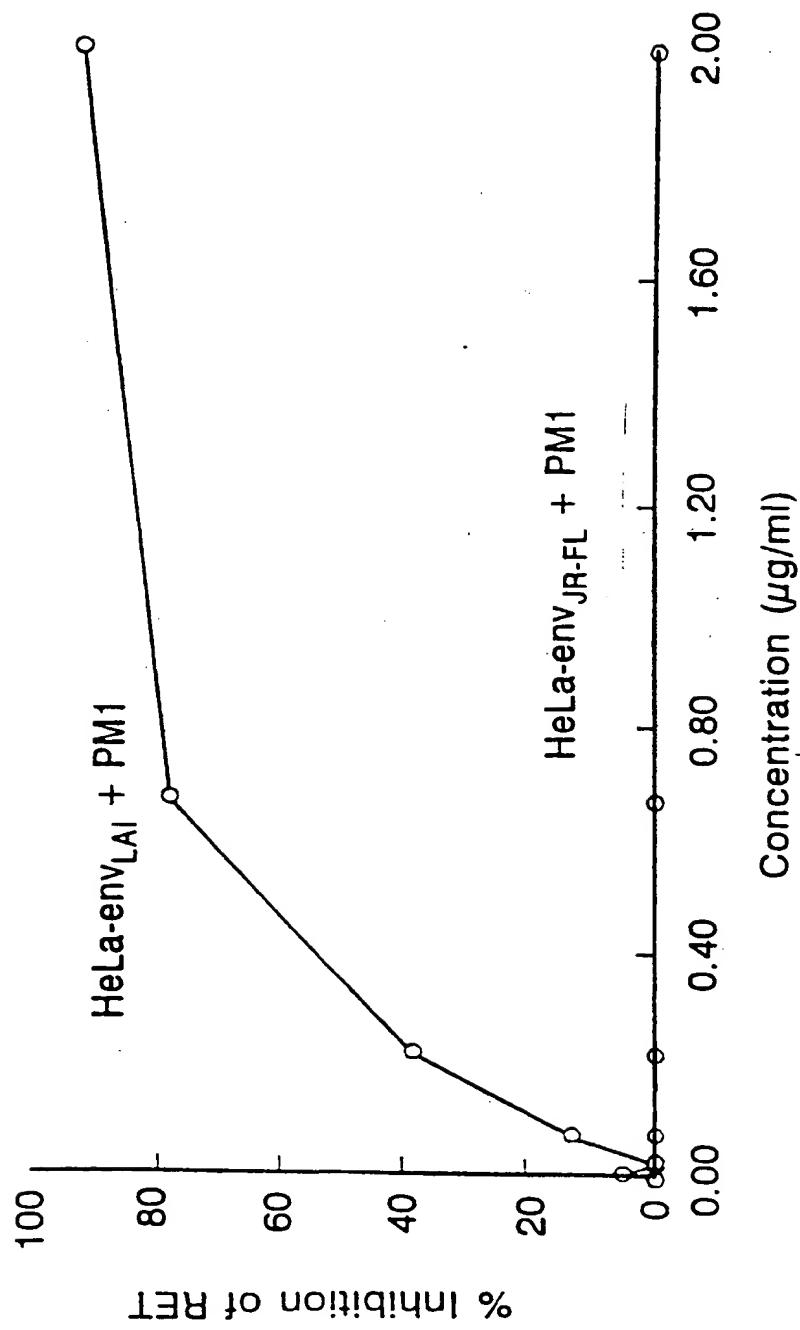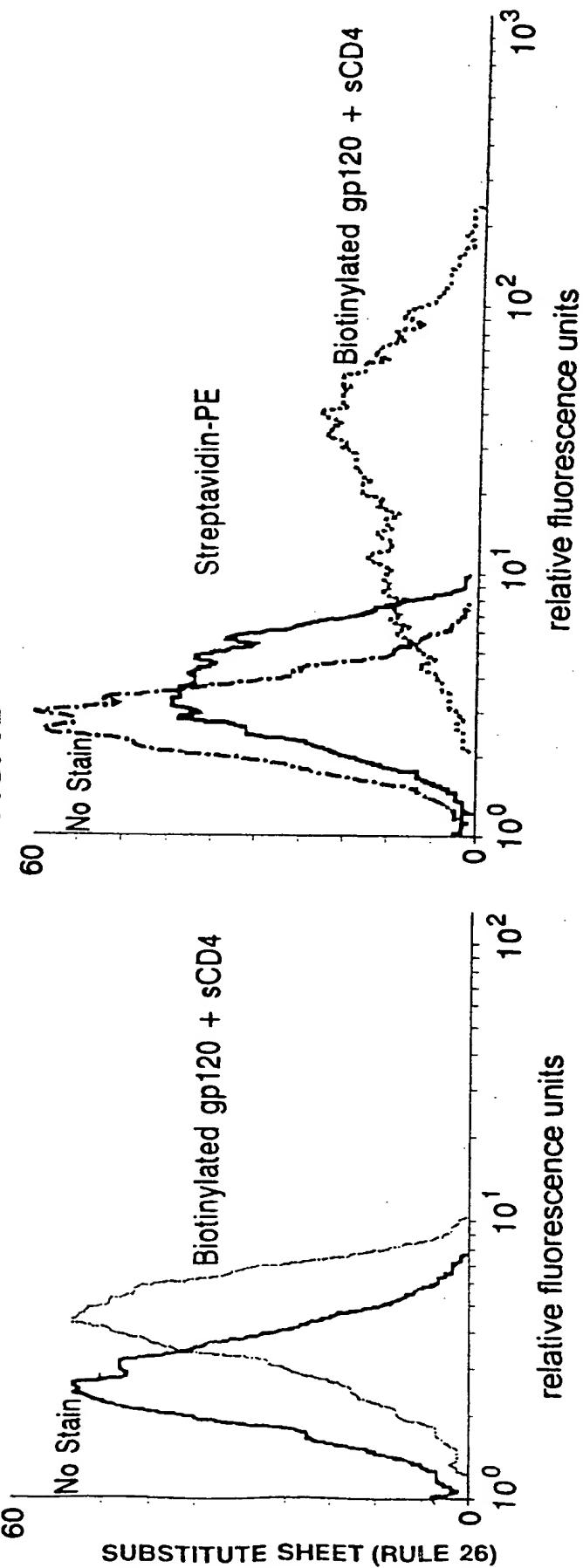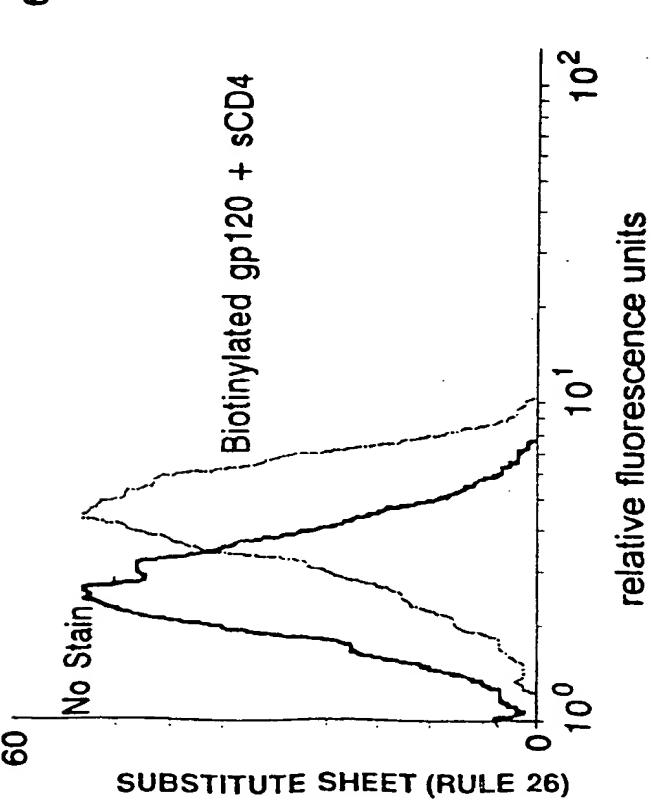
FIG. 7
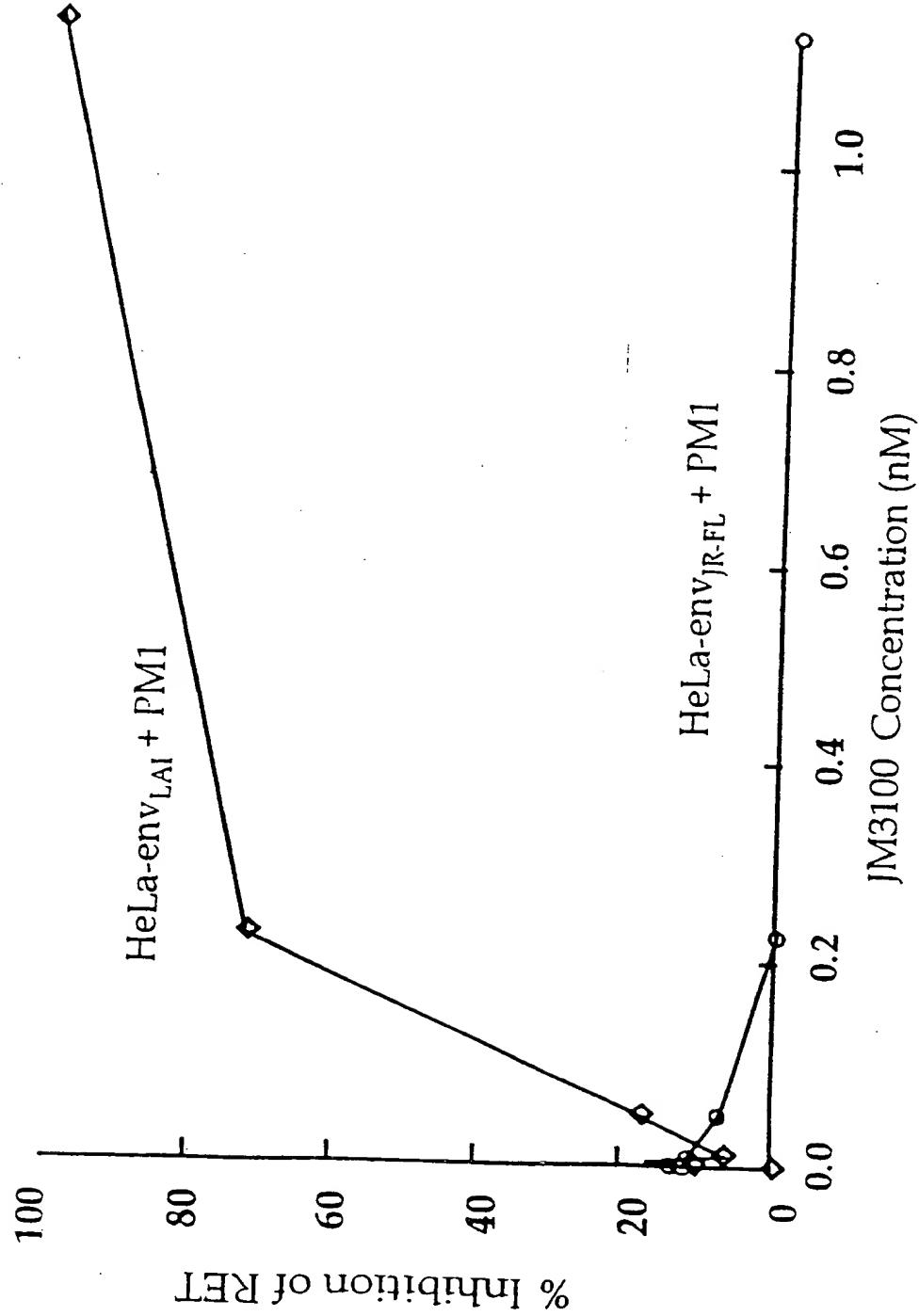
FIG. 8
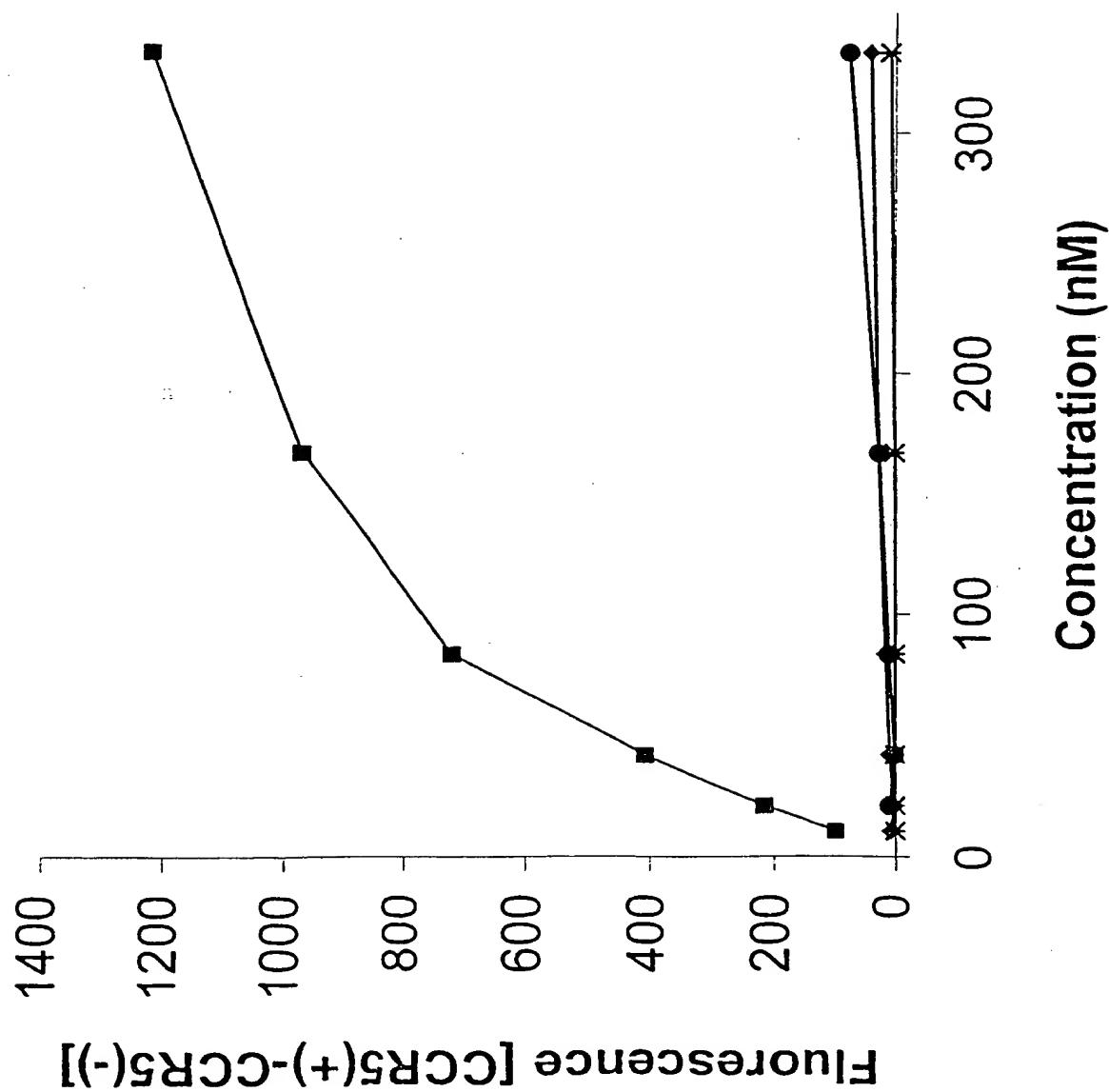
FIG. 9

Anti-CCR5 mAbs Inhibit gp120/CCR5 Binding
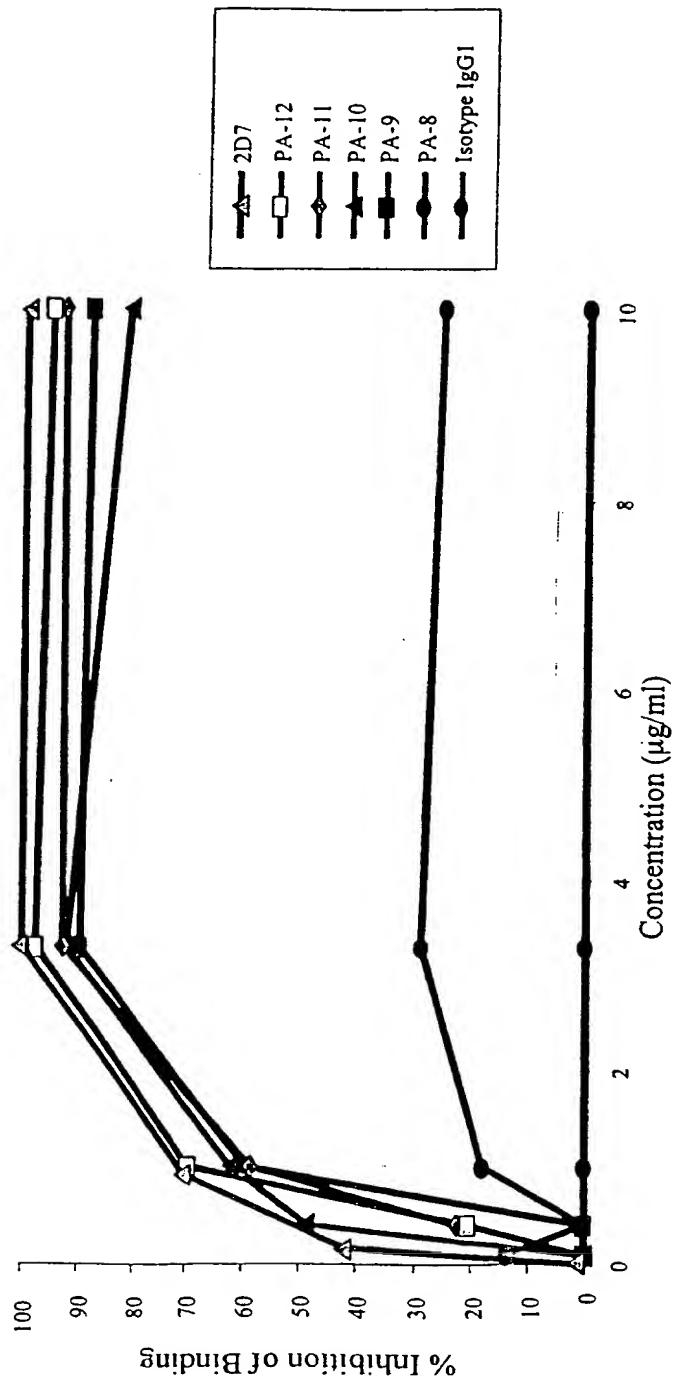
INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :Please See Extra Sheet.

US CL   :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	SIMMONS et al. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. Science. 11 April 1997, Vol. 276, pages 276-279, see entire document.	1-2, 4, 26, 32-33 ----------- 6-13, 27, 34-41, 43-54, 56-60
X -- Y	TRKOLA et al. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. Nature. 14 November 1996, Vol. 384, pages 184-187, see entire document.	1, 4, 26, 32-33 ----------- 2, 6-13, 27, 34-41, 43-54, 56-60

[X]	Further documents are listed in the continuation of Box C.	[ ]	See patent family annex.

* Special categories of cited documents:

"A"   document defining the general state of the art which is not considered to be of particular relevance

"E"   earlier document published on or after the international filing date

"L"   document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O"   document referring to an oral disclosure, use, exhibition or other means

"P"   document published prior to the international filing date but later than the priority date claimed

"T"   later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"   document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"   document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"   document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 SEPTEMBER 1998	1 5 OCT 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	ROBERT D. BUDENS
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ---- Y, P	DORANZ et al. A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. J. Exp. Med. 20 October 1997. Vol. 186, No. 8, pages 1395-1400, see entire document.	1-2, 4, 26, 32-33 ----------- 6-13, 27, 34-41, 43-54, 56-60
Y	COCCHI et al. Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8⁺ T cells. Science. 15 December 1995, Vol. 270, pages 1811-1815, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60
Y	FENG et al. HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science. 10 May 1996, Vol. 272, pages 872-877, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60
Y	DENG et al. Identification of a major co-receptor for primary isolates of HIV-1. Nature. 20 June 1996, Vol. 381, pages 661-666, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60
Y	OELLERICH, M. Enzyme-Immunoassay: A Review. J. Clin. Chem. Clin. Biochem. 1984, Vol. 22, No. 12, pages 895-904, see entire document, particularly Fig. 1.	34-41, 43-54, 56-60
Y, P	ALKHATIB et al. HIV-1 coreceptor activity of CCR5 and its inhibition by chemokines: Independence from G protein signaling and importance of coreceptor downmodulation. Virology. 09 August 1997, Vol. 234, pages 340-348, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60
A,P	SCARLATTI et al. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nature Medicine. November 1997, Vol. 3, No. 11, pages 1259-1265, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60
A	FAHEY et al. Status of immune-based therapies in HIV infection and AIDS. Clin. Exp. Immunol. 1992, Vol. 88, pages 1-5, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60
A	FOX, J. L. No winners against AIDS. Bio/Technology. February 1994, Vol. 12, page 128, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60
A	HAYNES et al. Update on the issues of HIV vaccine development. Ann. Med. 1996. Vol. 28, pages 39-41, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DAAR et al. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. Proc. Natl. Acad. Sci. USA. September 1990. Vol. 87, pages 6574-6578, see entire document.	1-2, 4, 6-13, 26-27, 32-41, 43-54, 56-60

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐   Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐   Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐   Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

     Please See Extra Sheet.

1. ☐   As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐   As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒   As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
   1-2, 4, 6-13, 26-27, 32-41, 43-54, and 56-60

4. ☐   No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☒   The additional search fees were accompanied by the applicant's protest.

                                   ☐   No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): A61K 45/05, 39/00, 39/385; C12Q 1/70; G01N 33/53, 33/536, 33/542, 33/567; C07K 14/52, 14/54, 17/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL : 424/85.1, 85.2, 184.1, 185.1, 193.1, 195.11, 198.1; 435/5, 7.2, 7.21, 7.24, 7.92, 7.93; 436/537, 542 ;
530/351, 395, 399, 402, 403

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/85.1, 85.2, 184.1, 185.1, 193.1, 195.11, 198.1; 435/5, 7.2, 7.21, 7.24, 7.92, 7.93; 436/537, 542 ; 530/351, 395, 399,
402, 403

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single
inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-27, 32-33, 51-52 and 56-60, drawn to non-chemokine agents.
Group II, claim(s) 28-32, drawn to compositions comprising non-chemokine agents linked to CD4.
Group III, claim(s) 34-50 and 53-55, drawn to methods for identifying non-chemokine agents.

This application contains claims directed to more than one species of the generic invention. These species are deemed to
lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In
order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as
follows:

I.   Species of non-chemokine agents as follows:

        Species A, drawn to oligopeptides and polypeptides (claims 2, 4, 6-23, 27, 32-33, 53-54);
        Species B, drawn to nonpeptidyl agents (claims 3, 25, 55);
        Species C, drawn to antibodies (claims 5, 24).

Should Applicant elect to pay for Species A set forth above, the following additional species lack unity of invention
under PCT Rule 13.1:

        Species A', drawn to non-chemokine agents which bind CCR5 (claims 6-13 and 41);
        Species B', drawn to non-chemokine agents which bind CXCR4 (claims 14-23, 42).

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under
PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I and
II are directed to products which differ in their physical and chemical properties such as composition and primary amino
acid sequence and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a
single general inventive concept. Further, the methods of groups I and III differ in their industrial applicability as well
as in their reagents and method steps and are not so linked by a special technical feature within the meaning of PCT
Rule 13.2 so as to form a single general inventive concept.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2,
the species lack the same or corresponding special technical features for the following reasons: The species are directed
to non-chemokine agents which also differ in their physical, chemical and biological properties and are not so linked by
a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.